Impact of helminths and helminth products on immune responses

A role for mast cells in orchestrating type 2 responses to helminths and a suppressive effect of a nematode immunomodulator in allergy

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M.Sc.-Ing. Biotechnologin Emilia Daniłowicz-Luebert

Präsident der Humboldt-Universität zu Berlin:
Prof. Dr. Jan-Hendrik Olbertz

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I:
Prof. Stefan Hecht, PhD

Gutachter:
1. Prof. Dr. Richard Lucius
2. Prof. Dr. Eckard Hamelmann
3. PD Dr. Uta Höpken

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3D	three-dimensional
AAM	alternatively activated macrophage
Ab	antibody
AD	atopic dermatitis
Ag	antigen
AHR	airway hyperreactivity
APC	antigen presenting cell
AR	airway reactivity
Arg	arginase
AvCystatin, Av17	filarial cystatin from *Acanthocheilonema viteae*
BAL	bronchoalveolar lavage
BAPNA	Nα-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride
BCA	bicinchoninic acid
BM	bone marrow
BME	β-mercaptoethanol
BMMCs	bone marrow-derived-cultured mast cells
CBP	carbohydrate based particles
CFSE	carboxyfluorescein succinimidyl ester
ConA	concavalin A
DC	dendritic cell
DHFR	dihydrofolate reductase
E/S	excretory/secretory
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin unit
FCS	fetal calf serum
Foxp3	forkhead box trasnscription factor p3
FPLC	fast performance liquid chromatography

GM-CSF	granulocyte-macrophage colony-stimulating factor
GPE	timothy grass pollen extract
H&E	hematoxylin and eosin
HCC	human cystatin C
HCF	human cystatin F
HDM	house dust mite
i.n.	intranasal
i.p.	intraperitoneal
ICAM	intercellular adhesion molecule
IFN	interferron
Ig	immunoglobuline
IL	interleukin
IPTG	isopropyl β-D-1-thiogalactopyranoside
ITIM	immunoreceptor tyrosine-based inhibition motif
KIT	tyrosine-kinase receptor
kUA/I	kilounits of antibody per liter
LCT4	leukotriene C4
LPS	lipopolysaccharide
LU	lab units
MC	mast cell
MCh	methacholine
MLN	mesenteric lymph node
mMCP	mouse mast cell protease
MPP	multipotent progenitor
MVE	maleic anhydride divinyl ether copolymer
MW	molecular weight
NBNT	non-B/non-T cell
NC	nitrocellulose (membrane)
NHC	natural helper cell
ORF	open reading frame
OVA	ovalbumine
p.i.	postinfection
PAF	platelet-activating factor

PALM	pollen associated lipid mediators
PAR-2	protease-activated receptor-2
PAS	periodic-acid Schiff
PBLN	peribronchial lymph nodes
PBMCs	peripheral blood mononuclear cells
PECs	peritoneal exudate cells
PFA	paraformaldehyde
Phl p 5b	major allergen group V isoform b
PMA	phorbol-myristate-acetate
PNU	protein nitrogen unit
PTMs	posttranslational modifications
RALS	right angle static light scattering
RELM	resistin-like molecule
RT	room temperature
s.c.	subcutaneous
SCF	stem-cell factor
SCIT	subcutaneous immunotherapy
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamid-gelelectrophoresis
SIT	allergen-specific immunotherapy
SLIT	sublingual immunotherapy
TBS	Tris-buffered saline
TGF	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
VCAM	vascular cell adhesion molecule
VLPs	virus-like particles
WHO	World Health Organization
WT	wild type
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

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Mojemu Kochanemu

Summary

Helminth infections induce protective type 2 (Th2) immune responses in the host leading to arrested larval development and/or intestinal worm expulsion. Moreover, Th2 immune responses are initiated against harmless environmental allergens and mediate a development of allergic disease.

Among multiple mechanisms implicated in host responses to parasites and allergens, mast cells (MCs) play a pivotal role. Until recently MCs were mainly considered as late-stage effector cells, whereas the contribution of MCs in the early events during Th2 immune response is relatively neglected. The present study shows that MC-deficient mouse strains following infection with two gastrointestinal helminths (*Heligmosomoides polygyrus* and *Trichuris muris*) had dramatically reduced early production of the tissue-derived cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which resulted in impaired induction of Th2 immune responses as well as increased parasite burden. Increased production of IL-25, IL-33, and TSLP, restored Th2 priming and reduced parasite burden was observed after 'repair' of the MC-compartment. These data reveal an important role for MCs in orchestrating type 2 immune responses.

Parasites have developed various strategies to modulate the immune system and ultimately suppress host protective Th2-type immune responses via the induction of a range of regulatory mechanisms. Additionally, treatment of inappropriate immune responses by the suppression of overwhelming inflammation by helminths has shown promise in initial clinical trials. For that reason, this work further explores the immunomodulatory role of the filarial cysteine inhibitor AvCystatin and its structural and functional characteristics in the murine model of airway hyperreactivity and inflammation induced by a clinically relevant allergen of timothy grass pollen. In this study AvCystatin was found to inhibit airway inflammation, airway hyperreactivity and to reduce grass pollen allergen-specific immune responses. At the same time AvCystatin increased levels of the regulatory cytokine IL-10 and numbers of $CD4^+CD25^+Foxp3^+$ T cells. Furthermore, the immunomodulatory effect *in vivo* was found to be independent of the protease inhibitor activity or oligomerization of AvCystatin.

The present study further aimed to evaluate the capacity of AvCystatin as a potential alternative adjuvant in an allergen-specific immunotherapy (SIT) using a novel murine model. It was found that AvCystatin did not exert additive or synergistic effects when applied during immunotherapy with the target antigen. Therefore, these data demonstrate that AvCystatin applied alone downregulates allergic inflammation and that its effect is not related to main cystatin's characteristics.

Finally, the aim was to translate the efficacious effects of AvCystatin seen in murine models to *in vitro* assays using human peripheral blood mononuclear cells (PBMCs) from grass pollen allergic patients. In the approach before the grass pollen season, AvCystatin suppressed allergen-specific production of IL-13 and created a shift towards Th1 immunity by increased levels IFN-γ. These findings suggest that AvCystatin may also have efficacy in the suppression of unwanted immune responses in disease patients.

Taken together the findings of the present study contribute to a better understanding of the early events that dictate the priming of type 2 immune responses, such as those raised during helminth infection or during allergies and, furthermore, show that helminths have co-evolved with the host to produce potent immunomodulatory products that act to suppress and modulate the Th2 response to ensure their survival. Heminth product-induced suppression may also have effects on bystander responses to unrelated antigens, thus, suggesting a promising preventive and therapeutic concept in the treatment of aberrant inflammations.

Zusammenfassung

Helmintheninfektionen induzieren in ihren Wirten schützende Typ 2 (Th2) Immunantworten, welche die Entwicklung von parasitären Larvenstadien hemmen und/oder zur Abstoßung intestinaler Würmer führen. Th2-Antworten können auch gegenüber harmlosen Umweltallergenen ausgebildet werden und vermitteln in diesem Zusammenhang die Entwicklung allergischer Erkrankungen.

Unter vielen Mechanismen, die für die Wirtsantwort gegen Parasiten und Allergenen von Bedeutung sind, spielen Mastzellen (MZ) eine herausragende Rolle. Bis vor kurzem wurden MZ hauptsächlich als Effektorzellen für die späte Phase der Immunantwort betrachtet. Einer möglichen Rolle von MZ für die frühe Phase von Th2 Immunantworten wurde bisher wenig Beachtung geschenkt. Die Ergebnisse der vorliegende Arbeit zeigen, dass eine Infektion von MZ-defizienten Mausstämmen mit gastrointestinalen Helminthen (*Heligmosomoides polygyrus* und *Trichuris muris*) zu einer dramatisch reduzierten Produktion von IL-25, IL-33 und Thymus-Stromal-Lymphopoietin (TSLP) sowie zu einer Beeinträchtigung der Etablierung von Th2 Immunantworten und einer erhöhten Wurmlast führt. Eine gesteigerte Produktion von IL-25, IL-33 und TSLP, wiederhergestellte Th2-priming Konditionen sowie eine verminderte Wurmlast konnte nach erfolgreichem Transfer von Knochenmark aus Wildtypmäusen beobachtet werden. Die vorliegenden Arbeit beschreibt damit eine wichtige Funktion von Mastzellen für die Initialisierung von Th2 Immunantworten.

Parasiten haben zahlreiche Strategien entwickelt um das Immunsystem ihrer Wirte zu modulieren und Th2 Immunantworten durch die Induktion regulatorischer Mechanismen zu supprimieren. Eine Behandlung von überschießenden Entzündungsreaktionen durch Helminthen wurde bereits durch erste klinische Studien demonstriert. Ein weiteres Ziel der vorliegenden Arbeit war es deshalb, die strukturellen und immunmodulatorischen Eigenschaften des Cystein-Protease-Inhibitors AvCystatin in einem murinen Modell für Atemwegsentzündung, induziert durch ein klinisch relevantes Allergen aus Graspollen, zu untersuchen. In diesem Zusammenhang konnte gezeigt werden, dass AvCystatin sowohl Parameter von Atemwegsentzündung und -hyperreaktivität als auch Graspollen-spezifische Immunantworten inhibiert. Gleichzeitig führte die Behandlung mit AvCystatin zu einem Anstieg von IL-10 und zu

erhöhten Frequenzen von $CD4^+CD25^+Foxp3^+$ T Zellen. Die *in vivo* Effekte von AvCystatin wurden dabei unabhängig von der Protease-inhibitorischen Aktivität des Immunmodulators oder einer Oligomerisation desselben vermittelt.

Des Weiteren sollte das Potential von AvCystatin als alternatives Adjuvans im Rahmen einer Allergen-spezifischen Immuntherapie (SIT) für ein neues Mausmodell untersucht werden. Dabei zeigte sich, dass eine Applikation von AvCystatin zusammen mit dem Zielantigen keine ergänzenden oder synergistischen Effekte vermittelt konnte. Diese Ergebnisse verdeutlichen, dass durch die Applikation von AvCystatin allergische Entzündungsreaktionen herunterreguliert werden und dass dieser Effekt unabhängig von der Protease-inhibitorischen Aktivität von AvCystatin vermittelt werden kann.

Ein weiteres Ziel der vorliegenden Arbeit war es, die Effekte von AvCystatin durch die Etablierung eines *in vitro* Systems mit mononukleären Zellen des peripheren Blutes (PBMCs) von Graspollen allergischen Patienten im humanen System zu untersuchen. In einem Versuchsansatz, der die Situation der Patienten vor der Graspollen-Saison widerspiegelt, unterdrückte AvCystatin die Allergen-spezifische Produktion von IL-13 und induzierte stattdessen eine Th1 Immunantwort durch die Induktion erhöhter Mengen von IFN-γ. Diese Ergebnisse zeigen, dass AvCystatin auch in humanen Graspollen-Allergikern unerwünschte Immunantworten unterdrücken kann.

Zusammenfassend tragen die Ergebnisse der vorliegenden Arbeit dazu bei, ein besseres Verständnis für die frühen Ereignisse von Th2 Immunantworten zu entwickeln, welche durch Helminthen oder Allergene induziert werden. Darüber hinaus bekräftigen die Daten die Hypothese, dass Helminthen im Rahmen einer langen Koevolution mit ihren Wirten effektive immunmodulatorische Strategien entwickelt haben, um die gegen sie gerichtete Th2-Antwort zu unterdrücken. Die Immunsuppression durch Helminthenmoleküle kann zugleich Bystandereffekte auf Immunantworten gegen andere Antigene vermitteln und birgt daher ein großes therapeutisches Potential.

1. Introduction

1.1 General aspects

Helminths have developed a unique evolutionary dialogue with their hosts' immune system due to multicellular nature, complex life cycles and their longevity within the host. These pathogens induce different immune responses than bacteria, fungi, viruses or protozoa do. Especially host mucosal epithelia are in close contact with helminths, common antigens and are important for initiation of type 2 (Th2) immunity that characterizes helminth infections as well as allergic reactions. Moreover, Th2 immune responses are also initiated against harmless environmental allergens and may lead to the development of allergic diseases.

Among multiple mechanisms implicated in host responses to parasites, mast cells (MCs) play a pivotal role. As tissue-based inflammatory cells they respond to signals of innate and adaptive immunity. Although classically considered as late-stage effector cells actively involved i.e., during host responses against parasitic helminths (reviewed in Pennock and Grencis, 2006), their innate role in orchestration of early stages of the Th2 immune responses is relatively overlooked. Sharing of critical roles in Th2-cell priming has been ascribed to tissue-derived cytokine signals, in particular IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (reviewed in Saenz et al., 2008). Thus, the question on the role of MCs in the regulation of the tissue-derived cytokines and subsequently the development of type 2 immunity remains unanswered.

Interestingly, parasites have developed various strategies to modulate the immune system and ultimately suppress host protective Th2-type immune responses e.g., by induction of innate and adaptive regulatory cells, anti-inflammatory cytokines and specific inhibitory antibody isotypes (Fig. 1.1; reviewed in Anthony et al., 2007; Daniłowicz-Luebert et al., 2011).

Helminth parasites are master regulators of immune responses in order to ensure life-long persistence in the host (Maizels and Yazdanbakhsh, 2003). Many studies of animal and human helminth infections have shown their potential for downregulating the immune system. Moreover, relevant epidemiological studies have observed that helminth-infected populations exhibit lower levels of immunopathological disorders such as Th1-related autoimmune diseases or aberrant Th2-related conditions

e.g., asthma or allergic rhinitis. These observations indicate an inverse global distribution of allergy and helminth infections. The first being an expanding problem of industrialized high-income countries, while the latter being a feature of developing countries (Fallon and Mangan, 2007; Yazdanbakhsh and Luty, 2011). The most promising strategy for treatment of allergic diseases is the suppression of overwhelming Th2 immunity and the induction of regulatory mechanisms (reviewed in Bosnjak et al., 2011; Jutel and Akdis, 2011).

This work is composed of two interconnected projects. It explores a role of elements of Th2 immunity in the development of early responses to helminths and investigates the potential of a single worm-derived molecule to downregulate Th2-type immunopathologies in allergic airway hyperreactivity and inflammation. The latter constitutes the core of this thesis.

1.2 Helminths and allergens induce type 2 immune responses

Helminth infections as well as allergic disorders represent complex innate and adaptive immune responses to foreign antigens leading, in most of the cases, to inflammatory reactions. In response to antigens and the context they are presented in, naive $CD4^+$ T cells can differentiate into four T helper (Th) cells subsets: Th1, Th2, Th17 and inducible regulatory T cells (iTregs) (Mosmann and Sad, 1996; Zhu et al., 2010; Wisniewski and Borish, 2011). Th2 cells play an important role in host immunity to extracellular parasites (e.g. helminths), which can impair larval development and adult worm feeding or leading to intestinal worm expulsion (Anthony et al., 2007; Herbert et al., 2009; Liu et al., 2010). They are also responsible for the development of bronchocontriction and airway remodeling leading to allergic disorders (Fujita et al., 2012).

During Th2 immune response, professional antigen presenting cells (APCs) process helminth antigens or allergens and display them to $CD4^+$ T cells that differentiate into polarised Th2 cells. Th2 cells function through production of various Th2-associated cytokines, such as interleukin (IL)-4, IL-5, IL-9, IL-10 and IL-13 and via homing to specific tissue compartments (Saenz et al., 2008; Lloyd and Hessel, 2010). Theses cytokines activate macrophages, eosinophils, and other immune cells including MCs . IL-4 and IL-13 induce differentiation of antigen-specific B cells and production of large amounts of antibodies (characteristically IgE) (Swain et al., 1990; Paul and Zhu, 2010). IgE immune complexes activate innate immune cells (like basophils and MCs) by cross-linking high affinity receptors (FcϵRI) on their surfaces.

Activated, sensitized and matured basophils and MCs degranulate, and release preformed mediators, including histamine, leukotrienes, proteases and prostaglandins. Presence of these secreted products results in smooth muscle constriction, vascular permeability, and further inflammatory cell recruitment (e.g., eosinophils and neutrophils) (Stone et al., 2010). Moreover, migration of Th2 cells to intestinal tissues and lungs effects in infiltration of eosinophils, via IL-5 production, and MCs, via IL-9. They are also capable to induce mucus production by goblet cells and smooth muscle remodeling.

All these mechanisms are protective against helminth infections and finally lead to larvae killing and/or intestinal worm expulsion (Finkelman et al., 1997). On the other hand, inappropriate activation of the Th2 immune response develops into airway inflammation and allergic disease progression leading to airway hyperreactivity (AHR) and in many cases to asthma. Moreover, two main phases are typical for Th2 responses in allergic reactions: the early phase where sensitization and the develop-

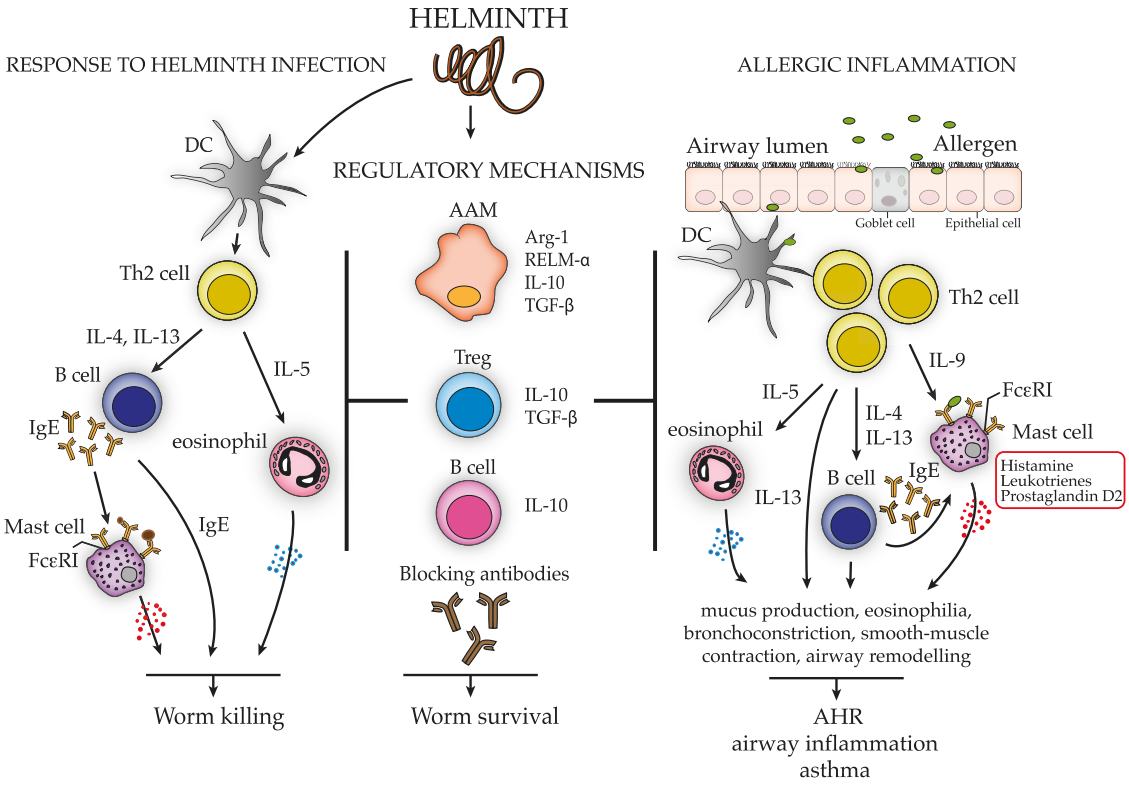

Figure 1.1: Helminths and allergens induce Th2 immune responses. Additionally helminth infections can generate regulatory mechanisms via modulation of immune cells leading to alternatively activated macrophages (AAMs), regulatory T cells (Treg), B cells and blocking antibodies (details in the text). These cellular changes lead to 'modified Th2' immune responses and larvae survival as well as blocking of unrelated inflammation such as allergic immune responses. Modified from Daniłowicz-Luebert et al. (2011).

ment of memory cells takes place, and the late phase when the effector cells lead to inflammation and tissue injury upon repeated exposure to the allergen (Akdis, 2006; Valenta, 2002). The early and late phases of Th2-type response in allergy are well studied. For that reason, the present work addresses early Th2 immune response induced by helminth infections where a role of MCs remains unclear.

1.2.1 Cytokines of early type 2 immune responses

Although later phases of Th2 immunity are well understood, the early events in Th2-type responses are relatively neglected.

Recent studies using Th2-associated disease animal models, reported on several novel innate immune cells and tissue-derived cytokines to be involved in shaping Th2 immune responses. Nuocytes (Neill et al., 2010), natural helper cells (NHCs) (Moro et al., 2010) and MPP^{type2} cells (Saenz et al., 2010) are necessary in the initiation of Th2 cascade. As Th2 priming happens at the tissue sites where allergens and parasites get in touch with e.g., skin, lung and gut tissue, 'early' tissue-derived cytokines such as IL-25, IL-33 and TSLP play a pivotal role.

Interleukin-25 is highly relevant in induction of Th2 immune responses. It has been described to be produced by Th2-polarized $CD4^+$ T cells, murine and human epithelial cells upon exposure to allergens (Fort et al., 2001; Angkasekwinai et al., 2007). Exogenous administration of IL-25 amplifies allergic type inflammatory responses with high expression of IL-4, IL-5 and IL-13, induces eosinophilia and increases IgE levels (Fort et al., 2001). In asthmatic mice IL-25 acts directly on $CD4^+$ T cells and promotes Th2-cell differentiation in an IL-4- and STAT6-dependent manner (Angkasekwinai et al., 2007). IL-25 also plays an important role in Th2-cell-mediated immunity to parasitic infections. Fallon et al. (2006) showed that mice lacking IL-25 have impaired *Nippostrongylus brasiliensis* worm expulsion and delayed Th2-associated cytokine production. IL-25, via regulation of non-B/non-T (NBNT), c-kit^+, FcϵRI-cells producing IL-4, IL-5 and IL-13, initiates worm expulsion (Fallon et al., 2006). Furthermore, Owyang et al. (2006) reported that *Trichuris muris*-infected genetically susceptible mice treated with exogenous IL-25 exhibited protective Th2 response to the infection.

Interleukin-33 induces differentiation of murine eosinophils (Stolarski et al., 2010), as well as activates human basophils and eosinophils of atopic patients (Pecaric-Petkovic et al., 2009; Suzukawa et al., 2008b,a). IL-33 acts, both alone or together with TSLP, on human primary MCs (Allakhverdi et al., 2007b). Upregulation of IL-33 is found in early phases of *T. muris* infection. Additionally, application of exogenous IL-33 leads to worm expulsion and increases TSLP expression within infected ceacum (Humphreys et al., 2008).

TSLP is overexpressed in lungs of allergic mice and by human epithelial cells from atopic patients (Zhou et al., 2005). It acts directly on $CD4^+$ T cells (Isaksen et al., 1999; Al-Shami et al., 2005), dendritic cells (DCs) (Soumelis et al., 2002) and MCs to increase Th2-cell responses (Allakhverdi et al., 2007a). Moreover, TSLP-activated DCs prime naive $CD4^+$ T cells to produce Th2 signature cytokines, such as IL-4, IL-5 and IL-13, and at the same time to downregulate IL-10 and IFN-γ (Soumelis et al., 2002). TSLP was reported to promote development of Th2 immune responses in infections with *T. muris* but not *Heligmosomoides polygyrus* or *N. brasiliensis* (Taylor et al., 2009; Humphreys et al., 2008).

In this context, IL-4 plays an essential role in communicating and differentiation of Th2 cells (Swain et al., 1990). Additionally, IL-4 upregulates the expression of GATA-binding protein 3 (GATA-3), the master regulator for Th2 differentiation and activator of genes encoding Th2-associated cytokines (Zheng and Flavell, 1997; Lee et al., 2000).

Although IL-25, IL-33 and TSLP are described to be mainly epithelial and endothelial cell-derived cytokines, recent studies identified alternative cell sources and various innate immune cells these cytokines act on (reviewed in Bartemes and Kita, 2012). Therefore it is important to further explore the role of mast cells in the regulation of these early type 2 immune responses.

1.2.2 Mast cells in parasite infections and allergy

Mast cells (MCs) are located at strategically important body barriers (such as skin, gut and lung tissue) and are recognized as important cells involved in Th2-associated immune responses. Their 'allergic activation' is initiated by allergens cross-linking of effector-cell-bound IgE via FcϵRI expressed on cell surface and leads to release of various pre-formed biologically active mediators (e.g., histamine, leukotrienes) (Rivera and Gilfillan, 2006). Late-phase reactions due to leukocytic and inflammatory (eosinophil, neutrophil and basophil) cell recruitment, lead to further cytokine production, tissue remodeling and in consequence to the development of type I hypersensitivity reactions (such as allergy, allergic asthma, rhinitis or urticaria) and later to the disease progression (reviewed in Moiseeva and Bradding, 2011).

The most commonly used animal model to study the role of MCs in immune responses is the WBB6F1-Kit^W/Kit^{W-v} or C57BL/6-Kit^{W-sh}/Kit^{W-sh} mouse. Both strains are profoundly deficient in MCs and melanocytes due to an improper transcription of the *kit* gene as a result of a mutation in the *W* locus. Due to the fact that Kit^W/Kit^{W-v} mice apart from their MC-deficiency have other abnormalities (such as anemia, sterility and almost complete lack of Cajal cells), it is often practiced to perform additional experiments in Kit^{W-sh}/Kit^{W-sh} mice that do not exhibit

those phenotypic abnormalities. The lack of MCs can be selectively repaired by the adoptive transfer of wild type, either whole bone marrow (BM) containing mast cell-progenitors or *in vivo*- or *in vitro*-derived MCs. BM or MCs can be administered in various ways, depending on a studied organ, induced infection or type of inflammation (reviewed in Metz et al., 2007).

In host defense against helminths, MCs contribute to elimination of established infection by affecting worm reproduction and survival. It has been known for many years that infections with nematodes such as *Trichinella spiralis* (Alizadeh and Murrell, 1984), *Strongyloides ratti* (Abe et al., 1993), *H. polygyrus* (Ben-Smith et al., 2003), *T. muris* (Betts and Else, 1999) and *N. brasiliensis* (Issekutz et al., 2001) correlate with a large mastocytosis, which affects later stages of the infection and accelerates adult worm (*T. spiralis*, *S. ratti* and *H. polygyrus*) expulsion from the intestine. Particularly mechanisms such as secretion of mucosal specific mast cell proteases (i.e., mMCP-1), increase of intestinal epithelial permeability or collaboration with Th2-cell-dependent mechanisms lead to MC-mediated worm expulsion (Knight et al., 2000; McDermott et al., 2003; Pennock and Grencis, 2006). MCs are also regarded as capable to serve as a source of various Th2-associated cytokines (such as IL-3, IL-5, IL-10, IL-13, IL-33 and TSLP), mediators (TNF-α, histamine, platelet-activating factor - PAF, leukotriene C4 - LCT4) that induce the expression of endothelial adhesion molecules (E-selectin, ICAM, VCAM-1) essential for lymphocyte homing (Mekori and Metcalfe, 1999; Henz et al., 2001; Saenz et al., 2008).

Only recent studies have begun to identify critical roles for MCs in the promotion of various immune responses to pathogens such as bacteria (Dietrich et al., 2010) or viruses (Wang et al., 2012) as well as in contact allergy (Dudeck et al., 2011). As MCs are located in a close proximity to DCs and epithelial cells, they can communicate via released mediators and cytokines, however the exact mechanism is still unknown. And although MCs are nowadays less considered only late-stage effector cells, their innate role during the initial events of the Th2 immune response during helminth infections remains relatively neglected.

1.3 Impact of helminths on prevalence of allergic reactions

Helminths induce regulatory mechanisms via modulation of immune cells. These mechanisms include alternatively activated macrophages (AAMs), regulatory T cells (Treg), B cells and production of blocking antibodies (IgG4 in humans, IgG1 in mice). AAMs in mice express various factors such as arginase-1 (Arg-1), resistin-like

molecule-α (RELM-α), IL-10, transforming growth factor-β (TGF-β) and contribute to wound healing. Treg produce IL-10 and TGF-β, whereas B cells can also elicit regulatory mechanisms via IL-10. Blocking antibodies compete with IgE for binding sites to worm antigens and minimize damage due to IgE-mediated response. These cellular changes lead to a 'modified Th2' immune response and larvae survival and can abolish unrelated inflammation such as allergic immune responses (Fig. 1.1) (reviewed in Maizels and Yazdanbakhsh, 2003; Holgate and Polosa, 2008).

1.3.1 Evidence from field studies

Eradication of helminth infections in industrialised countries in last decades is thought to have a great impact on the prevalence of diseases associated with inappropriate immune responses (reviewed in Fallon and Mangan, 2007). The observed increase in the appearance of allergy-related diseases might be a result of this and altered hygienic measures in everyday life. One possible explanation is the hygiene hypothesis (Strachan, 1989; Yazdanbakhsh et al., 2002), which names a number of factors like improved public health, use of antibiotics and vaccines that in consequence reduced the occurrence of viral, bacterial or helminth infections early in life, contributing to higher numbers of individuals with allergic and/or autoimmune disorders.

There are numerous cohort studies determining immune responses to environmental allergens of helminth-infected individuals in parasite-endemic areas (reviewed in Smits et al., 2010; Daniłowicz-Luebert et al., 2011). Particularly infections with trematodes, whipworms and hookworms were described to be negatively correlated with the allergen skin prick test against cockroach, house dust mites (HDMs) and common environmental aeroallergens (Araújo et al., 2000; Cooper et al., 2003; Medeiros et al., 2003; Flohr et al., 2009). Results from a study of Gabonese school children tested for skin reaction to HDMs and other allergens, showed lower prevalence of a positive skin test to HDMs in individuals with urinary schistosomiasis. Moreover, schistosome antigen-specific concentrations of IL-10 were significantly higher in infected children and concentrations of IL-10 were negatively associated with a positive skin test result (van den Biggelaar et al., 2000).

A direct contribution of active helminth infections to the inverse correlation with allergy-related diseases was demonstrated in anthelminthic treatment studies (Lynch et al., 1993; Endara et al., 2010; Flohr et al., 2010). Repeated treatment with anthelminthics in a population of Gabonese school children significantly increased the rate of developing skin sensitization to HDMs. Thus, the presence of live worms that suppress allergic responses during infection and prevent allergy later in life is important (van den Biggelaar et al., 2004). Also early exposures and infections

with helminths may have a protective effect and suppress allergic inflammation later during life (Huang et al., 2002; Rodrigues et al., 2008).

Additionally, host genetic factors may play a role in prevalence of helminth infections and allergies (Barnes et al., 2007; Scirica and Celedon, 2007; Smits et al., 2010).

In contrast to population studies reporting a negative correlation between allergy and helminth infections as discussed above, various studies from South America, Europe and Asia on *Ascaris lumbricoides* infections and allergy reported increased rather than decreased prevalence of asthma (Joubert et al., 1980; Dold et al., 1998; Palmer et al., 2002). It was proposed that heavy parasitic infections might generate immune suppressive mechanisms, whereas mild worm exposure and low-level contact with helminths may enhance reactivity to environmental allergens or even potentiate the Th2 immune response that in some cases may promote allergic inflammation (Dold et al., 1998). Additionally due to the specific pulmonary phase in the *Ascaris* life cycle that causes inflammation and eosinophilia (Enobe et al., 2006) and/or cross-reactivity of *Ascaris*-specific molecules (e.g., tropomyosin) with environmental allergens (HDMs, *Blomia tropicalis* that induce allergen-specific IgEs (Fernandes et al., 2003; Acevedo et al., 2011; Santiago et al., 2011), *Ascaris* infection has been well documented as a risk factor for asthma (Leonardi-Bee et al., 2006; Hagel et al., 2007; Alcantara-Neves et al., 2010).

Interestingly, the promotion of atopy can also be observed in parasitic infections where the human is not the definitive host e.g., *Toxocara* spp. (Gonzalez-Quintela et al., 2006; Flohr et al., 2009) or *Anisakis simplex* (Daschner et al., 2008). In this case the immune system may be exposed to Th2-inducing events rather than to immunomodulatory mechanisms that occur in chronic infections. In addition to field studies, experimental approaches in animal models are well reported.

1.3.2 Evidence from animal models of allergy

Due to the complexity of the population-association studies, animal models of allergy can complement the understanding on the effect and mechanism of individual helminth infections or more specifically, helminth-derived products on downregulating allergy-related diseases (Table 1.1).

Infection with male *Schistosoma mansoni* worms mediated resistance to ovalbumine (OVA)-induced AHR. The schistosome worm-infection prevented allergen-induced inflammation via IL-10-dependent suppression of pulmonary eosinophilia (Mangan et al., 2006). Moreover, infection with *H. polygyrus* was reported to suppress murine allergic asthma to both OVA and the HDM major allergen - Der p 1 (Wilson et al., 2005). Additionally, in a model of food allergy induced by

Table 1.1: Effects of helminth infections and helminth-derived products/molecules on improvement of symptoms in allergy-related experimental animal models.

	Allergy model	Suppressive mechanism	Reference
Helminth infections and undefined products			
S. mansoni infection; males and females; males	Pen V-induced systemic anaphylaxis	IL-10-producing B cells	Mangan et al. 2004
S. mansoni infection; males	OVA-induced AHR[a]	IL-10, B cells	Mangan et al. 2006
S. mansoni infection; eggs	OVA-induced AI[b]	IL-10-independent, $CD4^+CD25^+Foxp3^+$ Treg	Pacifico et al. 2009
S. japonicum egg antigen (SEA)	OVA-induced AI	$CD4^+CD25^+$ Treg, IL-10	Yang et al. 2007
A. suum adult worm extract (ASC)	OVA-induced AHR	NA[c]	Lima et al. 2002
H. polygyrus infection	OVA-induced AHR	IL-10	Kitagaki et al. 2006
	OVA-induced AHR	IL-10-independent, $CD4^+CD25^+$ T cells	Wilson et al. 2005
	HDM-induced AI	IL-10-independent, Breg cells	Wilson et al. 2010
	PN[d]-food allergy	IL-10	Bashir et al. 2002
H. polygyrus excretory/secretory product (HES)	OVA-induced AI	HES-induced Treg cells	Grainger et al. 2010
N. brasiliensis infection	OVA-induced AHR	IL-10	Wohlleben et al. 2004
N. brasiliensis excretory/secretory product (NES)	OVA-induced AI	IL-10-independent	Trujillo-Vargas et al. 2007
Strongyloides sterocalis infection	OVA-induced AI	NA	Wang et al. 2001
Strongyloides venezuelensis infection	OVA-induced AI	NA	Negrão-Corrêa et al. 2003
T. spiralis infection	OVA-induced AI	IL-10, TGF-β, $CD4^+CD25^+$ T cells	Park et al. 2011
L. sigmodontis infection	OVA-induced AHR	TGF-β, $CD4^+CD25^+$ Treg	Dittrich et al. 2008
Defined helminth-derived molecules			
smCKBP (*S. mansoni*)	Hapten-induced contact hypersensitivity	NA	Smith et al. 2005
Sm22.6, PIII, Sm29 (*S. mansoni*)	OVA-induced AI	$CD4^+Foxp3^+$ Treg	Cardoso et al. 2010
As-MIF (*A. simplex*)	OVA-induced AI	IL-10, TGF-β and Treg	Park et al. 2009
PAS-1 (*A. suum*)	APAS-3-induced AI	Possibly IL-10	Itami et al. 2005
	OVA-induced AHR	$CD4^+CD25^+$ T cell-, $CD8^+$ T cell-dependent and IL-10/TGF-β, IFN-γ-mediated	Araújo et al. 2008; de Araújo et al. 2010
Nippocystatin (NbCys) (*N. brasiliensis*)	OVA-inudced AI	Cathepsin B and cathepsin L-dependent	Dainichi et al. 2001b
AvCystatin (Av17) (*A. viteae*)	OVA-induced AHR	IL-10, macrophages, partially $CD4^+CD25^+$ T cell	Schnoeller et al. 2008
ES-62 (*A. viteae*)	Oxozolone-induced skin immediate hypersensitivity, OVA-induced AHR	MC-mediated, TLR-4-dependent	Melendez et al. 2007

[a] AHR - airway hyperreactivity
[b] AI - airway inflammation
[c] NA - data not known
[d] PN - peanut extract

peanut (PN), *H. polygyrus* infection decreased PN-specific IgEs, anaphylactic symptoms and secretion of IL-13 by PN-specific T cells (Bashir et al., 2002). Other helminths like *N. brasiliensis* or *Litosomoides sigmodontis* suppressed the development of OVA-induced airway eosinophilia and reduced the production of eotaxin in the airways of asthmatic mice either in IL-10- or in TGF-β- and Treg-dependent manner (Wohlleben et al., 2004; Dittrich et al., 2008).

Strikingly, a pre-existing helminth infection with *Strongyloides stercoralis* increased systemic OVA-induced Th2-type responses while at the same time reduced levels of allergen-specific IgE and eotaxin levels in bronchoalveolar lavage (BAL) fluid after exposure to the allergen (Wang et al., 2001). Also infection with *H. polygyrus* in a murine model of OVA-induced atopic dermatitis, led to the aggravation of allergen-induced inflammation and recruitment of MCs into eczematic regions. Production of allergen-specific IgEs, infiltration of $CD8^+$ and $CD4^+$ T cells into the skin was reduced with unaltered numbers of Treg cells (Hartmann et al., 2009).

It is evident that the protective role of helminth infections on allergic reactions depends on the type of parasitic infection and on investigated experimental model of allergic disease. Published reports help understanding the mechanism of helminth immunomodulation of non-related inflammation and shed some light on the potential of specific helminth-derived products that should be further evaluated.

1.4 Impact of helminth-derived products and defined molecules on allergy models

Studies from helminth infections in experimental animal models where infection-induced suppression of allergen specific Th2 responses is observed, suggest that products that are actively or passively released by the parasites may mediate the immuneregulation. Table 1.1 reviews described helminth-derived products and defined molecules and their mechanism of action in interference with allergy-related symptoms.

For example the helminthic excretory/secretory (E/S) of *H. polygyrus* (HES) was shown to bear TGF-β-like activity and to directly drive forkhead box trasnscription factor p3 (Foxp3) expression in $CD4^+$ T cells. Adoptively transferred HES-induced Treg cells suppressed OVA-induced allergic airway inflammation *in vivo*, as well as effector cell proliferation *in vitro* (Grainger et al., 2010). The E/S product of *N. brasiliensis* (NES) inhibited the development of eosinophilia, goblet cell metaplasia in the lungs and the development of AHR. NES decreased levels of OVA-specific IgG1 and IgE in serum, as well production of IL-4 and IL-5 in the airways in IL-10-independent manner. Interestingly, some non-protein components of NES were

found to be responsible for its suppressive effects (Trujillo-Vargas et al., 2007).

Another, apart from AvCystatin, well-defined molecule from *Acanthocheilonema viteae*, ES-62, was described to promote differentiation of DCs and macrophages to anti-inflammatory phenotype (Goodridge et al., 2004). The molecule interfered with a mouse model of immediate-type hypersensitivity to oxazolone and diminished ear swelling, as well as reduced degranulation of MCs. In the same report authors provided evidence that ES-62 molecule ameliorated OVA-induced airway inflammation, airway hyperresponsiveness, lung pathology and eosinophilia, as well as decreased production of IL-4 via suppression of MC-function (Melendez et al., 2007).

Although several helminth-derived molecules are described to interfere with antigen-dependent immune reactions, cysteine protease inhibitors (cystatins) are well-characterized protease inhibitors that are not only involved in biological processes but have also immunomodulatory potential.

1.5 Cystatins

Cystatins are inhibitors of cysteine proteases of the papain-like family and represent an important tool for regulation of potentially harmful activities of proteases present in the body (reviewed in Turk et al., 2008).

1.5.1 Cystatins - classification and structural characteristics

Cystatin superfamily encompasses proteins that contain multiple cystatin-like sequences and based on the primary sequence homology, it is composed from three families: stefins (family 1), cystatins (family 2) and kininogens (family 3) (Abrahamson et al., 2003).

Cystatins typically possess an N-terminal signal peptide and two conserved internal disulfide bonds. However, filarial cystatins lack the second disulphide bridge as the second C-terminal cysteine residue pair around PW is missing. A further structural characteristic is the existence of a conserved glycine residue (GG) within the N-terminal region of the protein that may function as a hinge between the flexible N-amino-terminal segment and the rest of the molecule (Hall et al., 1998).

The N-terminal region with the conserved glycine residue forms - together with a central Q-X-V-X-G motif and a C-terminal PW hairpin loop - the cysteine protease interaction site, which directs the cystatin molecule into the active site cleft of the cysteine protease (Bode et al., 1988). All three regions form a hydrophobic wedge-like structure, which is highly complementary in shape and mediates protease inhibition (Fig. 1.2).

Under physiological conditions cystatins form monomers (Turk et al., 1985; Ekiel and Abrahamson, 1996), however some cystatins form dimeric or oligomeric structures that reduce the inhibitory function of the protein (Staniforth et al., 2001; Kotsyfakis et al., 2010).

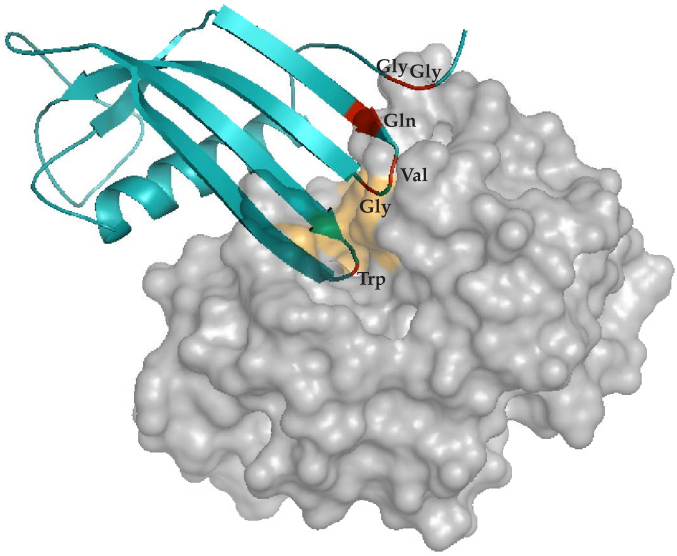

Figure 1.2: Predicted model of the AvCystatin-papain complex. AvCystatin is shown in cyan and papain in gray with its active site cleft in orange. The N-terminal conserved Gly-Gly (GG), Gln-X-Val-X-Gly (Q-X-V-X-G) and Trp (W) motifs (red) of AvCystatin fill papain's active site cleft along the whole length. Papain PDB code: 9PAP (Kamphuis et al., 1984), AvCystatin was modeled using ESyPred3D (Lambert et al., 2002).

Up-do-date there is no report on posttranslational modifications (PTMs) of parasitic cystatins. However, another *A. viteae*-derived glycoprotein ES-62 was described to bear a PTM that apparently plays an important role in immunomodulatory characteristics of this molecule (Goodridge et al., 2004).

One of the aims of this work was to produce three forms of AvCystatin (mutated, monomeric and demethylated AvCystatin's derivates) in order to study the influence *in vivo* of inhibitory activity and structural characteristics (oligomerization) as well as an approach to explore a possible involvement *in vitro* of PTMs on the immunomodulatory performance of AvCystatin.

1.5.2 Cysteine protease inhibitors of parasites

Cystatins from parasitic organisms represent proteolytic enzymes that may interfere, modulate or even suppress host immune effector mechanisms. A cystatin from *Brugia malayi Bm*-CPI-2 was illustrated to interfere with antigen processing, which led to

a reduced number of epitopes presented to T cells *in vitro* (Manoury et al., 2001). *L. sigmodontis* at various stages of the life cycle secretes a cystatin, which after injection via micro-osmotic pumps into the peritoneal cavity of *L. sigmodontis*-infected mice greatly decreased nitric oxide production and proliferation of antigen-specific spleen cells (Pfaff et al., 2002). The recombinant cystatin from the E/S product of *N. brasiliensis* (nippocystatin, NbCys) inhibits cathepsins L and B, and suppresses antigen processing by APCs (Dainichi et al., 2001a). *In vitro* studies on cystatin from human filarie *O. volvulus* (Ov17) revealed that the molecule interfered with mitogen- and antigen-induced stimulation of human peripheral blood mononuclear cells (PBMCs) and led to secretion of high amounts of IL-10 and inhibition of T cell proliferation (Schönemeyer et al., 2001; Hartmann et al., 2002). An arthropod-derived sialostatin L from a parasite *Ixodes scapularis* with anti-cathepsin L activity, exhibits anti-inflammatory role and inhibits proliferation of cytotoxic T lymphocytes contributing to tick-feeding success (Kotsyfakis et al., 2006). Two protease inhibitors suppressing non-related immune reactions are included in the Table 1.1.

1.5.3 Filarial cystatin - AvCystatin

One of the best-described cystatins of parasites is a filarial cysteine protease inhibitor from *A. viteae* with molecular weight of 17kDa, AvCystatin (cystatin, Av17). Previous studies showed AvCystatin to be a potent modulator of macrophages and to induce anti-inflammatory and anti-proliferative immune responses *in vitro* (Hartmann et al., 1997, 2002; Schierack et al., 2003). AvCystatin is recognized by macrophages (Hartmann et al., 1997; Klotz et al., 2011b). Furthermore, AvCystatin administered intraperitoneally (i.p.) in an OVA-induced asthma model, suppressed recruitment of eosinophils into the lungs, total and OVA-specific IgE levels and reduced OVA-specific IL-4 production by induction of IL-10-producing macrophages (Schnoeller et al., 2008).

This thesis investigates properties and further clinical potential of AvCystatin in an experimental model of allergy.

1.6 Experimental models to study allergies

In order to explore treatment strategies of allergy and asthma and to propose new therapeutic interventions, the development of improved animal models using clinically relevant allergens is necessary.

Up-to-date the most commonly used mouse model of allergic airway hypereactivity is an OVA-induced model, where OVA is a classical, inexpensive, widely available allergen. However, OVA while being a model allergen that does not cause allergic

reactions in every-day life, it bears certain limitations in animal models such as the quite possible induction of tolerance and therefore alleviation of airway inflammation (Wiedermann et al., 1999; Fuchs and Braun, 2008). Moreover, repeated airway administrations with OVA were reported to induce a progressive reduction of asthmatic symptoms in sensitized mice (Schramm et al., 2004; van Hove et al., 2007). For this reason it is essential to work with improved animal models of allergic airway hyperreactivity and inflammation that use clinically relevant inhalant allergens such as HDMs or pollens that humans are allergic to (Herz et al., 2004).

Several groups report on mouse models using human relevant allergens either as major recombinant allergens or natural extracts, such as house dust mite allergen (rDer f l) and cockroach allergen (rBla g 2) (Sarpong et al., 2003), recombinant or natural olive pollen allergen (rOle e 1, nOle e 1) (Batanero et al., 2002), birch pollen allergen (rBet v 1) (Wiedermann et al., 2001), timothy grass pollen allergen (rPhl p 5) (Linhart et al., 2007), rye grass pollen extract (Cadot et al., 2010) or *Aspergillus fumigatus* extract (Murdock et al., 2012). Depending on many factors (e.g., usage of adjuvants, mice strain, ways of administration) efficiency of clinically relevant allergens to activate the immune response and induce allergic symptoms vary (reviewed in Torres et al., 2005). Thus, considering above, timothy grass pollen was used in the present study to establish a mouse model of allergic airway hyperreactivity and inflammation.

Grass pollen allergens are the most prevalent in causing allergic disorders. It is known that around 40% of allergic patients are sensitized to grass pollens with timothy grass (*Phleum pratense*) pollen being the most common allergen in Europe with harmful effects in the season from April to October (Andersson and Lidholm, 2003). Its major allergen group V isoform b (Phl p 5b) is responsible for more than 90% of allergic activity in grass pollen-sensitized patients (Petersen et al., 1993; Flicker et al., 2000).

This study evaluates whether a mouse model of asthma can be established using recombinant Phl p 5b and if the resulting airway hyperreactivity and inflammation can be ameliorated using the helminth immunomodulator AvCystatin.

1.7 Allergen-specific immunotherapy

Recently more popular to study in animal models and one of the most promising treatment strategies of allergy in humans is an immunemodifying therapy - 'allergen-specific immunotherapy (SIT)' giving reasonably promising results, exists since 101 years. In SIT repeated exposure to allergen(s) leads to immunological tolerance, production of blocking antibodies IgG4 in humans, allergen-specific IgAs, potentially

regulatory cytokine IFN-γ and induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells that are a source of regulatory IL-10 and/or TGF-β. These mechanisms attenuate allergen-specific Th2-cell responses, including MCs, basophils, eosinophils, allergen-specific IgEs and Th2-associated cytokines (reviewed in Jutel and Akdis, 2011), and were initially described by Platts-Mills et al. (2001) as 'modified Th2' responses in cat-allergic children.

Currently practiced subcutaneous administration of allergen extracts or recombinant allergens as subcutaneous immunotherapy (SCIT) gives good results and long-term efficacy (Durham et al., 1999). Moreover, clinical trials with children showed that SCIT not only decreased symptoms of allergy, but also reduced development of asthma and might prevent new sensitizations in rhinitis (Möller et al., 2002; Jacobsen et al., 2007). An alternative to SCIT is a sublingual immunotherapy (SLIT), which implies administration of higher doses of allergens to the oral mucosa with very mild side-effects. In 2009, based on a review of more than 60 controlled trials, a World Health Organization (WHO) confirmed the safety and clinical efficacy of SLIT (Canonica et al., 2009).

Despite the advances in SIT over a century, decreased immunogenicity, improved efficacy and safety are the main aims of research on SIT nowadays. This includes addition of omalizumab; usage of T-cell-reactive peptides, hypoallergenic recombinant allergens, chemically modified allergens; embedding allergens into nanoparticles; supplementing with immunostimulatory DNA sequences (CpG) or novel adjuvants with decreased side-effects (reviewed in Casale and Stokes, 2011).

In frame of this work, the potential of AvCystatin as an alternative adjuvant was evaluated.

1.8 Treatment strategies of allergy and asthma

Incidence of allergic diseases is increasing and have already reached epidemic proportions worldwide. Thus, it is critical to identify new small-molecules and biological interventions, as well as improve existing treatment strategies that efficiently tackle the 'allergic cascade'.

One of the existing strategies proposed to combat allergies is 'allergen avoidance'. Although used as primary prophylaxis, it gives disappointing results because e.g. the complete elimination of contact with allergens is not possible in all cases. Available established treatments are mainly based on symptom relieving (e.g., corticosteroids, β-adenoreceptor agonists, mediator antagonists such as cetirizine or loratadine and leukotriene modifiers) (reviewed in Holgate and Polosa, 2008). Additionally, few therapeutics influencing IgEs are being tested, with the most successful so far - oma-

lizumab, a humanized IgE-specific, non-anaphylactic IgG1 that has been developed for severe IgE-mediated allergic asthma (Holgate et al., 2005). New therapeutic approaches to inhibit MC-activation via the modulation of Fc-receptor signaling, as well as drug candidates targeting stem-cell factor (SCF) with its tyrosine-kinase receptor (KIT), which is necessary for MCs development, are currently being evaluated (reviewed in Malbec and Daëron, 2007).

Further on, application of biological agents such as blocking monoclonal antibodies, different fusion proteins or inhibitors of Th2-cell transcription factors aims to modulate Th2 cells and their responses that orchestrate development of allergic inflammation. Some of the targets has successfully completed one or more phases of clinical trials in asthma (Bosnjak et al., 2011).

In order to assess whether AvCystatin is able to exert its immunomodulatory effect not only in the pre-clinical murine model but also in the human system, the effect of AvCystatin on peripheral blood mononuclear cells (PBMCs) isolated from clinically characterized, timothy grass (*P. pratense*) pollen allergic patients was tested in frame of this thesis.

2. Aim of the study

Led by the above discussed findings, the aim of this study was to test the following hypotheses:

1. One component of the early events in Th2 immunity are innate immune cells. It is predicted that in helminth infections mast cells (MCs) are particularly involved in early responses, priming and establishment of type 2 immunity.

2. The helminth immunomodulator AvCystatin was shown to reduce OVA-induced allergic asthma in mice. Therefore, depending on its structure (monomers/oligomers) and biological function as cysteine protease inhibitor, AvCystatin should suppress timothy grass pollen-specific allergic responses by controlling excessive Th2 immune reaction.

3. AvCystatin was shown to downmodulate allergy through reduction of inflammation and induction of regulatory mechanisms. The hypothesis that AvCystatin can serve as an alternative adjuvant, which enhances the effect of treatment with allergen-specific immunotherapy was evaluated.

Results of this dissertation are presented in five sections.

Section 3.1 explores a role of MCs in early events of parasitic infection using two helminths (*H. polygyrus* and *T. muris*).

Section 3.2 evaluates the effect of a single helminth molecule AvCystatin in a mouse model of allergy induced by timothy grass pollen.

Section 3.3 analyzes the relevance of structural and functional aspects of AvCystatin in the immunomodulatory effect.

Section 3.4 examines AvCystatin as an adjuvant in allergen-specific immunotherapy.

Section 3.5 assesses the capacity of AvCystatin on modulation of timothy grass pollen-induced allergic responses in a human setting.

3. Results

3.1 The role of mast cells during Th2 immunity in helminth infection

3.1.1 Impaired Th2 responses and reduced host protection following intestinal helminth infections during mast cell deficiency

Mast cells (MCs) are well described as effector immune cells and their central role in Th2-type immune responses (e.g. allergy) is well recognized. Moreover, MCs are now considered major immune cells involved in host defense, and no longer solely as 'allergy cells'. Although vast knowledge on the role of MCs in allergy, it is unknown whether MCs contribute to the early events of non-allergic conditions, as helminth infections that induce Th2-type immune reactions.

In order to address this question, an influence of MCs during infection with two model gastointestinal parasites was studied. *H. polygyrus* is a small intestinal dwelling helminth that can establish Th2-type chronic infection and *T. muris* - a caecal dwelling nematode associated with Th1/Th2 response.

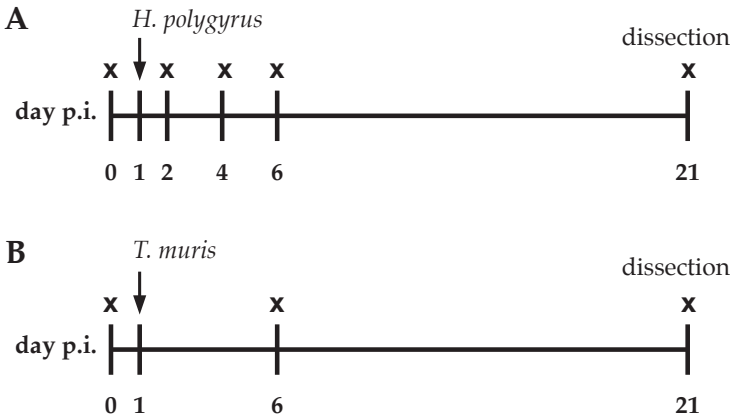

Figure 3.1: Scheme of infection with (A) *Heligmosomoides polygyrus* and (B) *Trichuris muris*. Animals were infected at day 1 with L3 *H. polygyrus* larvae or embryonated *T muris* eggs. Immunological parameters were analyzed at day 0 (naive animals), days 2, 4, 6 and 21 postinfection (p.i.) for *H. polygyrus*-infected animals or at days 0, 6 and 21 p.i. for *T. muris*-infected animals.

To this end, two MC-deficient mice strains (Kit^{W}/Kit^{W-v} and Kit^{W-sh}/Kit^{W-sh}) and corresponding wild type (WT) (WBB6 and C57BL/6) animals were infected with *H. polygyrus* (*Hp*) and *T. muris* (*Tm*) (Fig. 3.1). Early events and mechanisms involved in the immune response to infections were analyzed.

Infection with the duodenal helminth *Heligmosomoides polygyrus*

In order to test the influence of MCs on the cellular responses during *H. polygyrus* infection, levels of IL-2 and *Hp*-derived antigen (*Hp*-Ag) induced proliferation were evaluated in WT and MC-deficient mice. Analysis at an early time point (day 6) as well as later at day 21 post infection (p.i.) showed that MC-deficient mice had reduced draining lymph node hypertrophy, significantly lower levels of *Hp*-specific IL-2 detected in the draining mesenteric lymph node cell (MLNs) culture supernatants and had weakened proliferation of MLN cells to *Hp*-Ag when compared with WT control mice (Fig. 3.2A, B). As expected, increased levels of mMCP-1 were observed only in serum of WT mice but not of MC-deficient mice (Fig. 3.2C) as assessed at day 4 and 6 p.i. This proves MCs activity in WT mice only.

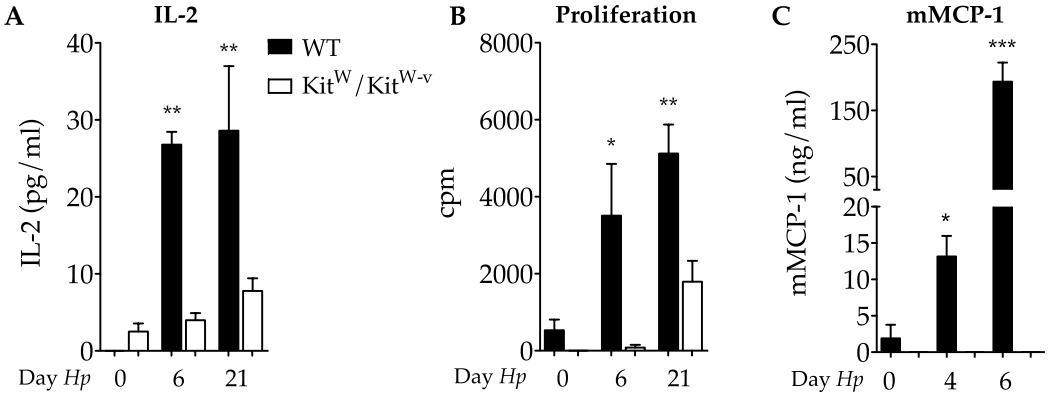

Figure 3.2: MC-deficient mice lack proper Th2-type cellular responses. (A) IL-2 production and (B) proliferation of MLNs in response to *H. polygyrus* antigen in naive animals (day 0) and day 6 and 21 p.i. with *Hp*; (C) serum concentrations of mMCP-1. Compared with naive controls. The mean values ±SEM are shown for 6-8 mice per group, representative data from one out of six independent experiments. *, $p< 0.05$; **, $p< 0.01$ are considered statistically significant.

Moreover, analysis at day 21 p.i. revealed that MC-deficient mice harbored significantly increased numbers of viable adult worms compared with WT controls (Fig. 3.3A). Consequently, MC-deficient mice showed significantly increased parasite egg counts in the faeces, which according to the literature is likely due to lack of effector activity of MCs (Fig. 3.3B). Increased levels of Th2-associated cytokines (IL-4, IL-5, IL-9, IL-10 and IL-13) in response to *Hp*-Ag stimulation were detected at day 6 and 21 p.i. in MLNs culture supernatants of WT control mice only.

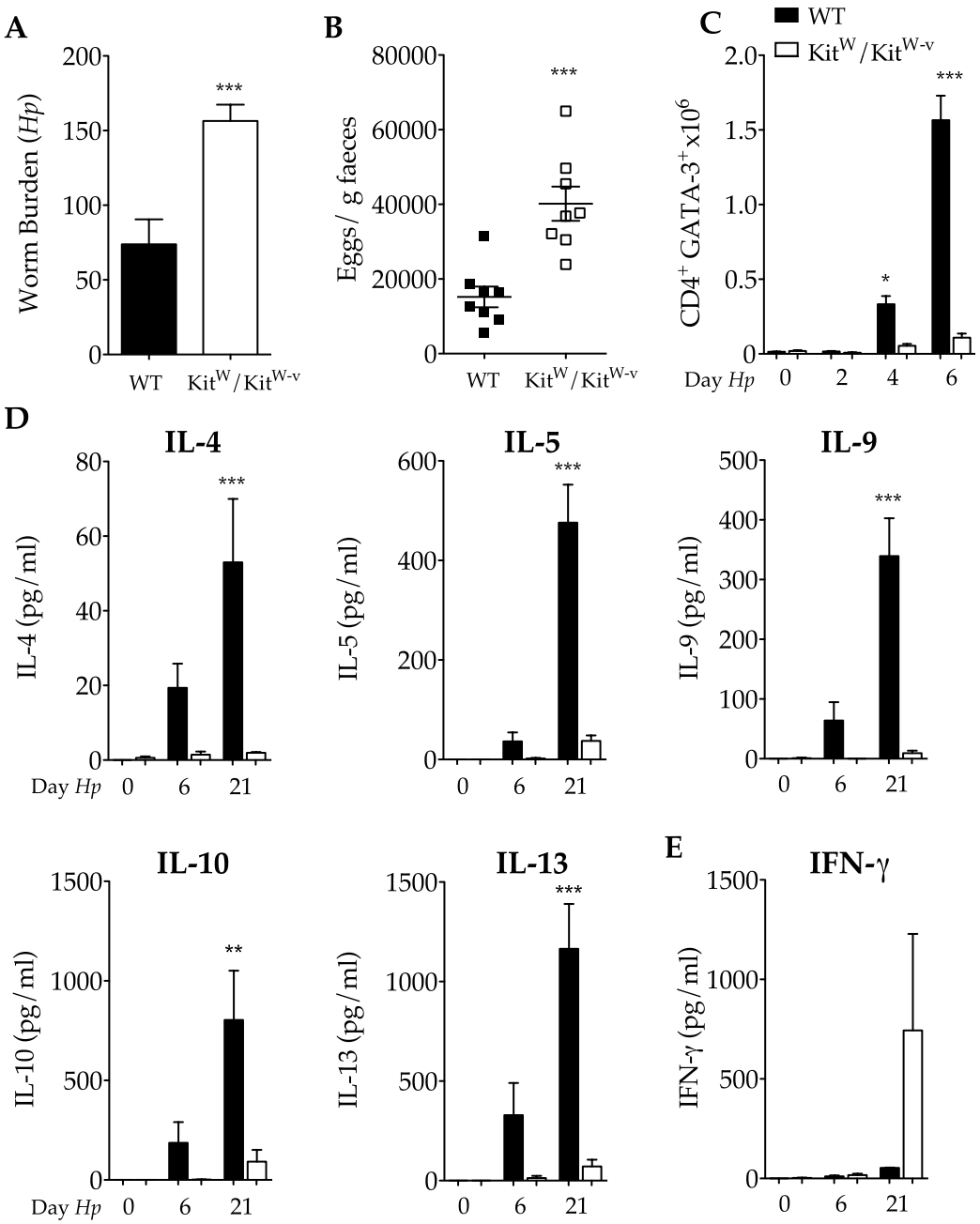

Figure 3.3: Increased parasite burden after *H. polygyrus* infection and impaired Th2-type responses of MC-deficient mice. (A) Adult worm numbers at day 21 p.i.; (B) median (pooled) parasite egg output per gram faeces of three independent measurements per mouse assessed on day 14, 16 and 18 p.i.; (C) total MLN cell numbers of GATA-3$^+$ CD4 $^+$ Th2 cells at days 0, 2, 4, and 6 after *H. polygyrus* infection; (D) and (E) *H. polygyrus*-specific cytokine production by MLNs. Compared with naive mice. The mean values ±SEM are shown for 6-8 mice per group, representative data from one out of six independent experiments. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$ are considered statistically significant.

Moreover, a strong induction of the Th2 transcription factor GATA-3 in $CD4^+$ T cells in the MLN of WT mice was observed by day 4 p.i. In addition, MC-deficient mice had significantly lower frequency of GATA-3^+ cells in comparison with WT animals at day 4 p.i. (0.7% ± 0.1% vs. 3.0% ± 0.4%, respectively) (Fig. 3.3C). This demonstrates a strong Th2-polarised response of WT mice, in contrast to significantly impaired Th2 priming of MC-deficient mice (Fig. 3.3D). On the other hand, MC-deficient mice exhibited variable increased production of IFN-γ at 21 p.i. (Fig. 3.3E).

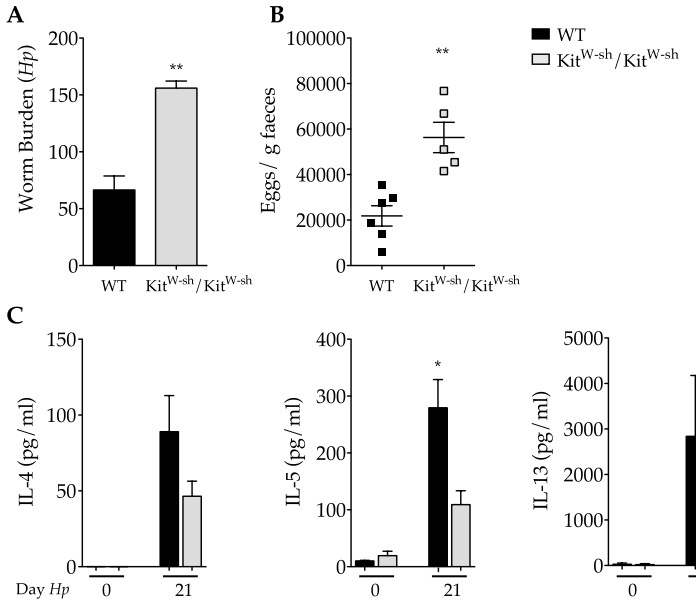

Figure 3.4: Kit^{W-sh}/Kit^{W-sh} mice have a lack in Th2-type response and increased parasite burden after *H. polygyrus* infection. (A) *H. polygyrus* worm numbers analyzed at day 21 p.i.; (B) median (pooled) parasite egg output per gram faeces of three independent measurements on day 14, 16 and 18 p.i.; (C) *Hp*-Ag induced IL-4, IL-5 and IL-13 production in MLN cell culture supernatants from naive animals (day 0) and animals at day 21 p.i. Compared with (A, B) WT mice, (C) naive mice. The mean values ±SEM are shown for 5 mice per group, representative data of one out of two independent experiments. *, $p< 0.05$; **, $p< 0.01$ are considered statistically significant.

Mast cell-deficient mice (Kit^W/Kit^{W-v}) due to their mutation in the white spotting (W) locus (i.e. c-kit) exhibit not only lack of MCs but also a high incidence of other abnormalities (e.g., anemia and sterility) and spontaneous pathologies affecting the skin, stomach or duodenum. In contrast, Kit^{W-sh}/Kit^{W-sh} mice, caring the W-sash (W^{W-sh}) inversion mutation, have MC-deficiency but lack anemia and sterility. Thus, in frame of this work, it was important to confirm the findings from *H. polygyrus*-infected Kit^W/Kit^{W-v} mice in these Kit^{W-sh}/Kit^{W-sh} mice.

Analysis of Kit^{W-sh}/Kit^{W-sh} mice to worm burdens, egg output in faeces and cytokine responses at the site of the infection as well as in the supernatants of

MLNs restimulated with *Hp*-Ag confirmed the observed impaired Th2-type immune responses to this parasite in the former model of MC-deficiency (Fig. 3.4).

Therefore, in line with reports on other intestinal nematode infections, it can be concluded that lack of MCs in two independent mice strains impairs a development of Th2-type immune response during *H. polygyrus* infection.

Infection with *Trichuris muris* in mast cell-deficient mice

Additionally, Kit^{W-sh}/Kit^{W-sh} mice were infected with the second intestinal helminth - a ceacal dwelling *T. muris* inducing a mixed Th1/Th2-type immune response.

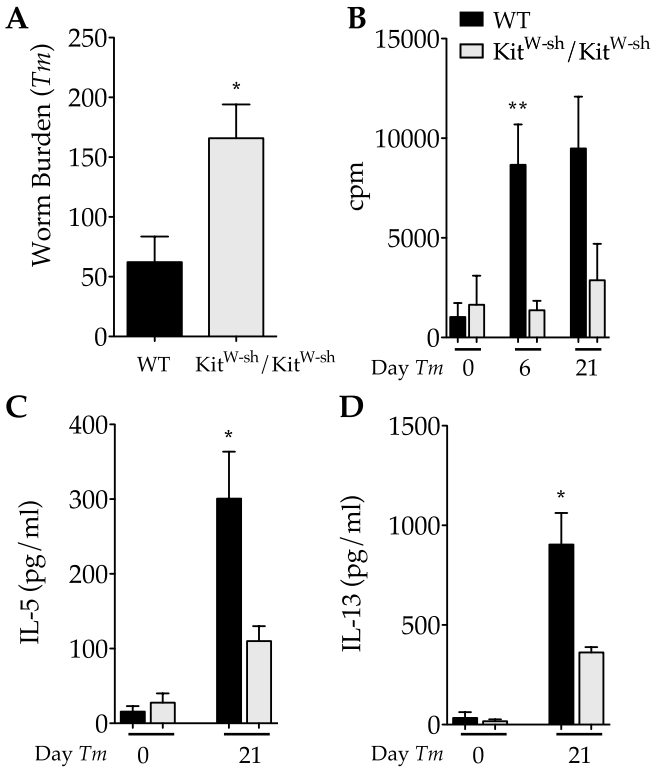

Figure 3.5: *T. muris*-infected Kit^{W-sh}/Kit^{W-sh} mice lack proper Th2 immune responses. (A) *T. muris* worm burdens in the caecum and colon at day 21 p.i.; (B) Proliferation of MLNs restimulated with *Tm* excretory/secretory (E/S) antigen (Ag) in naive controls (day 0) and after 6 and 21 days p.i.; (C) and (D) *Tm* E/S-Ag induced IL-5 and IL-13 production in MLN cell culture supernatants from naive animals (day 0) and animals at day 21 p.i. Compared with (A) WT mice and (B-D) naive mice. The mean values ±SEM are shown for 7-8 mice per group, data of two pooled independent experiments. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$ are considered statistically significant.

MC-deficient mice showed an impaired induction of Th2 responses to *T. muris* infection, which was characterized by significantly higher parasite burdens at day 21 p.i. when compared with WT mice (Fig. 3.5A).

T. muris infection of Kit^{W-sh}/Kit^{W-sh} MC-deficient animals resulted in significantly reduced proliferation of MLNs restimulated with *Tm* E/S antigen (Ag) (a gift from R. Grencis, effective restimulation with E/S Ag was established in the group of R. Grencis) (Fig. 3.5B). Moreover, these animals had decreased levels of E/S-Ag-specific IL-5 and IL-13 measured in MLN cell culture supernatants at day 21 p.i. (Fig. 3.5C, D). This indicates that MC-deficiency impairs proper development of Th2 immune responses to *T. muris*.

3.1.2 Requirement of IL-25 for proper Th2 immune responses during helminth infection and mast cell deficiency

IL-25, IL-33 and TSLP are critical 'early' Th2-type tissue-derived cytokines and are required for progenitor expansion and subsequent Th2 priming. Thus, the possibility existed that an impaired Th2 immune response during the helminth infection in MC-deficient mice is a consequence of reduced production of these cytokines.

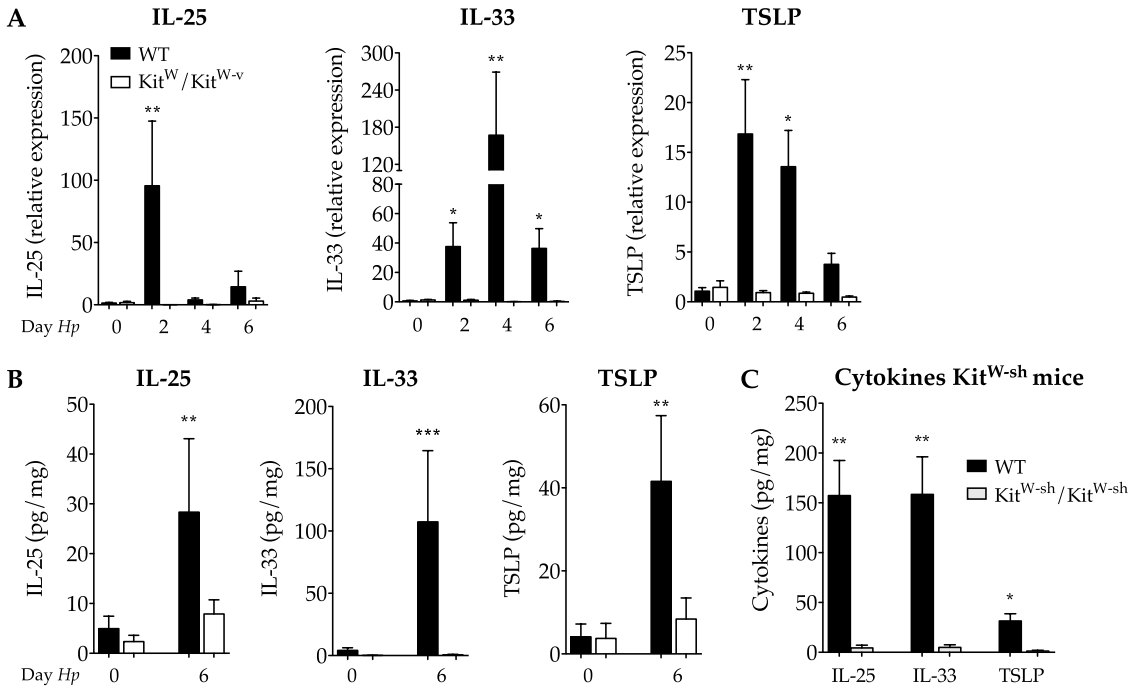

Figure 3.6: Tissue derived cytokines are expressed at early days of *H. polygyrus* infection. (A) IL-25, IL-33 and TSLP mRNA expression in naive animals (day 0) and at days 2, 4 and 6 post *H. polygyrus* infection. (B) Protein levels of IL-25, IL-33 and TSLP in duodenal homogenates (normalised to total tissue homogenate protein content) in naive mice and at day 6 p.i. in Kit^{W}/Kit^{W-v} mice and (C) at day 6 p.i. in Kit^{W-sh}/Kit^{W-sh} mice. Compared with (A-B) naive mice, (C) WT mice. The mean values ±SEM are shown for 7-8 mice per group, representative data of one out of three independent experiments.*, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$ are considered statistically significant.

In order to verify this hypothesis, production of these cytokines at the site of infection was assessed at early time points of *H. polygyrus* infection in WT and Kit^W/Kit^{W-v} mice.

Indeed, as assessed by real-time PCR on duodenal sections at days 2, 4 and 6 p.i. with *H. polygyrus*, mRNA levels of IL-25, IL-33 and TSLP were significantly increased over levels of naive animals (day 0) in WT mice but not in MC-deficient mice (Fig. 3.6A).

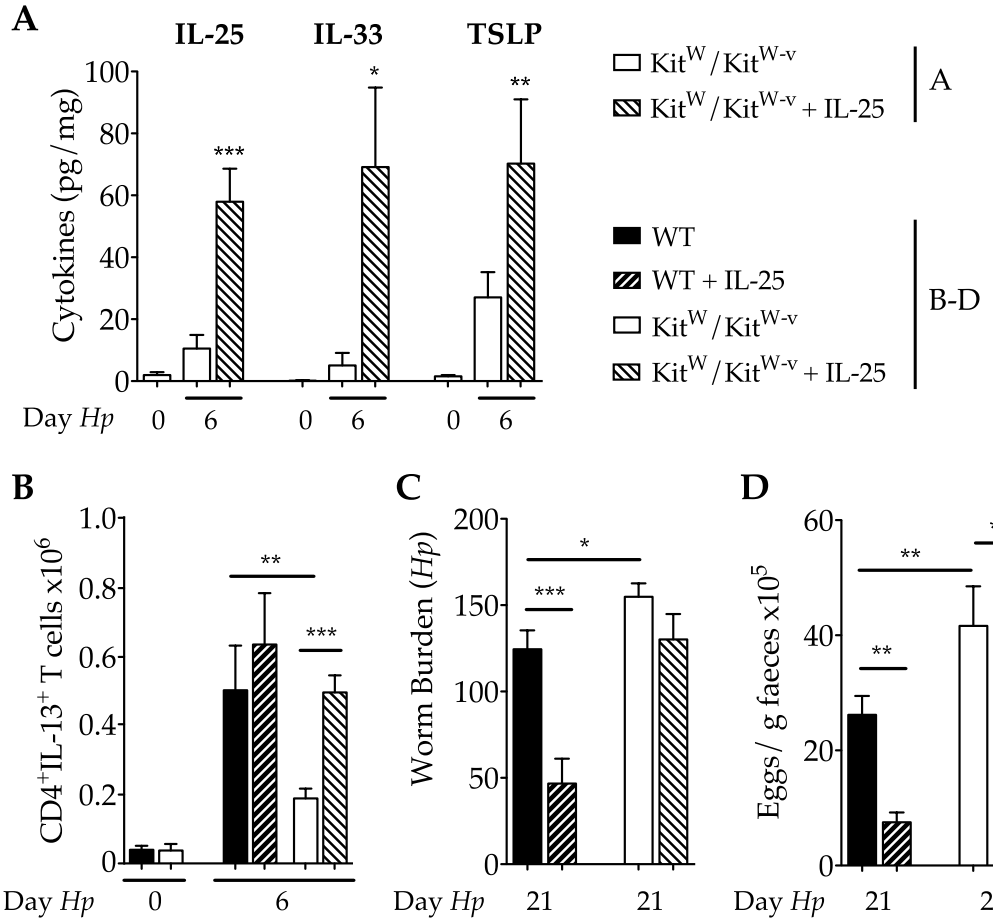

Figure 3.7: Early administration of rIL-25 induces anti-helminth immunity in the absence of MCs. (A) IL-25, IL-33 and TSLP protein levels in duodenal homogenates of naive animals (day 0) and at day 6 p.i. with *H. polygyrus* after rIL-25 application in Kit^W/Kit^{W-v} mice; (B) total numbers of $CD4^+IL\text{-}13^+$ T cells in the MLNs of naive (day 0) and at day 6 of *Hp*-infected WT, Kit^W/Kit^{W-v}, and rIL-25-treated mice; (C) adult worm burdens assessed at day 21 p.i. of *Hp*-infected WT, Kit^W/Kit^{W-v}, and rIL-25-treated mice; (D) median (pooled) egg output of *Hp*-infected WT, Kit^W/Kit^{W-v}, and rIL-25-treated mice. Compared accordingly with WT and MC-deficient controls. The mean values ±SEM are shown for 6 mice per group, pooled data from three independent experiments. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$ are considered statistically significant.

Moreover, all three cytokines were detected at protein level in gut homogenates of WT, but not MC-deficient animals at day 6 p.i. (Fig. 3.6B). Similarly, results were confirmed in a second MC-deficient mouse strain (Kit^{W-sh}/Kit^{W-sh}), where production of IL-25, IL-33 and TSLP in gut homogenates was also found to be reduced at day 6 p.i. with either *H. polygyrus* (Fig. 3.6C) or *T. muris* (data not shown). This suggests that MCs play a role in regulation of tissue-derived cytokines and in return, in induction of Th2 immune responses.

As IL-25 was found to be expressed as the earliest cytokine and in the strongest level at day 2 p.i. with *H. polygyrus* (Fig. 3.6A), it was interesting to determine whether exogenous application of IL-25 was sufficient to restore proper Th2 responses in Kit^{W}/Kit^{W-v} MC-deficient mice.

To verify this, recombinant IL-25 was applied i.p. to MC-deficient mice during the first 4 days of infection. Indeed, administration of rIL-25 dramatically increased expression not only of IL-25 but also of IL-33 and TSLP in the intestine at day 6 p.i. (larval stage of *H. polygyrus*) in comparison with untreated Kit^{W}/Kit^{W-v} control infected mice (Fig. 3.7A). Treatment with rIL-25 significantly restored priming of $CD4^{+}IL\text{-}13^{+}$ T cells in the MLNs to levels comparable with WT animals (Fig. 3.7B). Additionally, assessment of adult worm burdens at day 21 p.i. (adult worms stage) showed that application of rIL-25 did not change worm counts in MC-deficient mice. However, it led to almost complete expulsion of adult worms in WT mice, which suggests that very high amounts of IL-25 may induce worm expulsion during a normally chronic infection (Fig. 3.7C). Surprisingly, although worm expulsion was not affected in *H. polygyrus*-infected MC-deficient and rIL-25-treated mice, shedding of parasite eggs into the faeces was significantly reduced in comparison to untreated Kit^{W}/Kit^{W-v} controls (Fig. 3.7D). This points into the crucial role of early IL-25 in the development of anti-helminth immunity, which is characterized by impaired *H. polygyrus*-egg production in MC-deficient mice.

3.1.3 Transfer of wild type bone marrow restores Th2 immunity and anti-parasitic responses in mast cell-deficient mice

To further define the role of MCs in the orchestration of Th2 responses during *H. polygyrus* infection, it was crucial to restore the MC-compartment of mucosal tissues in Kit^{W}/Kit^{W-v} mice. The MC-compartment was restored via transfer of whole bone marrow (BM) from WT animals as described in the literature (Sakata-Yanagimoto et al., 2011). Although BM transfer results also in the transfer of other cells' progenitors, however due to the inability of MCs-transfer to properly reconstitute the small intestine, this method has been accepted in numerous publications.

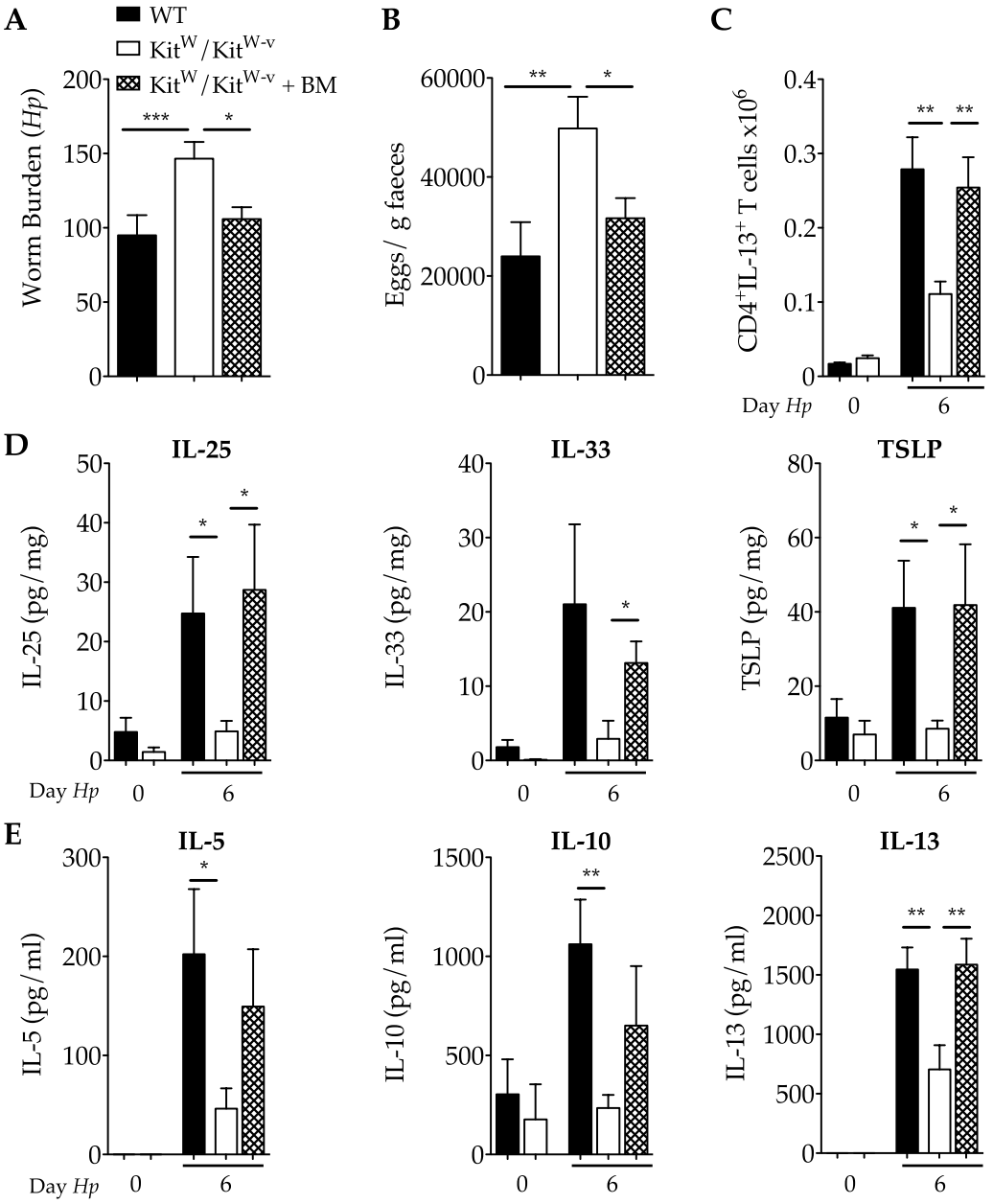

Figure 3.8: 'Repair' of MC-populations in Kit^{W}/Kit^{W-v} mice via transfer of bone marrow (BM) from WT mice restores proper Th2 immune responses and reduces *H. polygyrus* parasite burden. (A) Adult worm burdens at day 21 p.i.; (B) median (pooled) egg output per gram faeces; (C) total numbers of CD4^{+}IL-13^{+} T cells; (D) protein levels of IL-25, IL-33 and TSLP in duodenal homogenates; (E) levels of IL-5, IL-10 and IL-13 in *Hp*-Ag restimulated MLN cell culture supernatants in naive animals (day 0) and at day 6 p.i. of WT, Kit^{W}/Kit^{W-v} and BM reconstituted Kit^{W}/Kit^{W-v} animals. The mean values ±SEM are shown for 6 mice per group, pooled data from three independent experiments. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$ are considered statistically significant.

H. polygyrus-infected Kit^W/Kit^{W-v} animals that had been 'repaired' for MC-deficiency via adoptive transfer of WT BM showed a significant reduction of worm burdens and eggs shedding to faeces as assessed at day 21 p.i., comparable to WT infected controls (Fig. 3.8A, B).

BM transfer also led to restoration of IL-25, IL-33 and TSLP levels in the duodenum following *H. polygyrus* infection in comparison to infected, non-reconstituted control MC-deficient mice at day 6 p.i. (Fig. 3.8D). Furthermore, an increase in the total cell numbers in MLNs of $CD4^+IL\text{-}13^+$ T cells was observed in BM reconstituted *H. polygyrus*-infected Kit^W/Kit^{W-v} mice and levels were comparable with WT mice (Fig. 3.8C). BM reconstituted mice had markedly enhanced *Hp*-Ag-specific production of IL-5, IL-10 and IL-13 detected in the MLNs culture supernatants (Fig. 3.8E).

These data indicate, that MCs play a crucial role in Th2 priming and in the innate regulation of the tissue-derived cytokines IL-25, IL-33 and TSLP during the early stages of immune responses in the intestine during helminth infection. Hence, mice deficient in MCs developed a reduced early Th2-type response that could be restored by reconstitution with exogenous IL-25 and bone marrow transfer. These data provide novel information regarding the innate role of MCs in the development of Th2 immune response. It is tempting to speculate that possibly MCs communicate via the production of adjuvant signals during the early stages of gastrointestinal helminth infections.

3.2 Establishment of a murine model of allergic asthma with a clinically relevant allergen and treatment with AvCystatin

3.2.1 Grass pollen- versus OVA-induced model

In order to establish a mouse model of allergic airway hyperreactivity and inflammation induced by a clinically relevant allergen, the recombinant timothy grass (*Phleum pratense*) pollen allergen group V isoform b (rPhl p 5b) was used to sensitize mice and timothy grass pollen extract (GPE) was used for intranasal challenges.

After a literature review on published mouse models of allergy, in frame of the present work three intraperitoneal (i.p.) sensitizations with with rPhl p 5b/Alum and two intranasal (i.n.) challenges with GPE were applied to BALB/c mice (Fig. 3.9A). In parallel, a classical widely-used OVA-induced allergy model was performed where animals were sensitized i.p. two times with OVA/Alum and challenged twice i.n. with OVA (Fig. 3.9B).

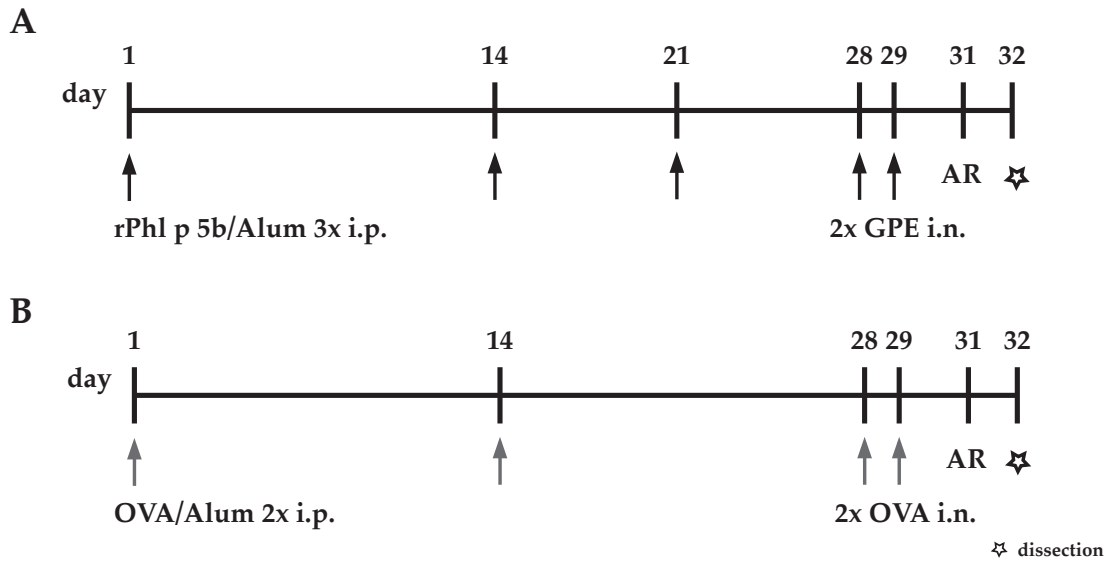

Figure 3.9: Scheme for allergen-induced mouse model of allergic airway hyperreactivity. (A) rPhl p 5b-induced model: animals were injected three times i.p. with rPhl p 5b/Alum and challenged two times i.n. with whole grass pollen extract (GPE). (B) Ovalbumine (OVA)-induced model: mice were sensitized twice i.p. with OVA/Alum and challenged twice i.n. with OVA. Naive control animals were treated with PBS/Alum and challenged with PBS. Measurement of airway reactivity (AR) was performed on day 31 and the dissection was done one day later.

Possibly due to the nature of the allergen and number of sensitizations, the rPhl p 5b-induced model exhibits several parameters stronger when compared with an OVA-induced classical model.

In order to evaluate the induction of main hallmarks of asthma like systemic sensitization, airway inflammation and induced airway hyperreactivity (AHR), composition of inflammatory cells in the BAL, cytokine production and airway reactivity was analyzed. In the rPhl p 5b-induced model local immune responses were characterized by a significant infiltration of inflammatory cells into the lungs, which was predominated by eosinophils. The eosinophil and lymphocyte numbers were higher than in OVA-induced model (Fig. 3.10A).

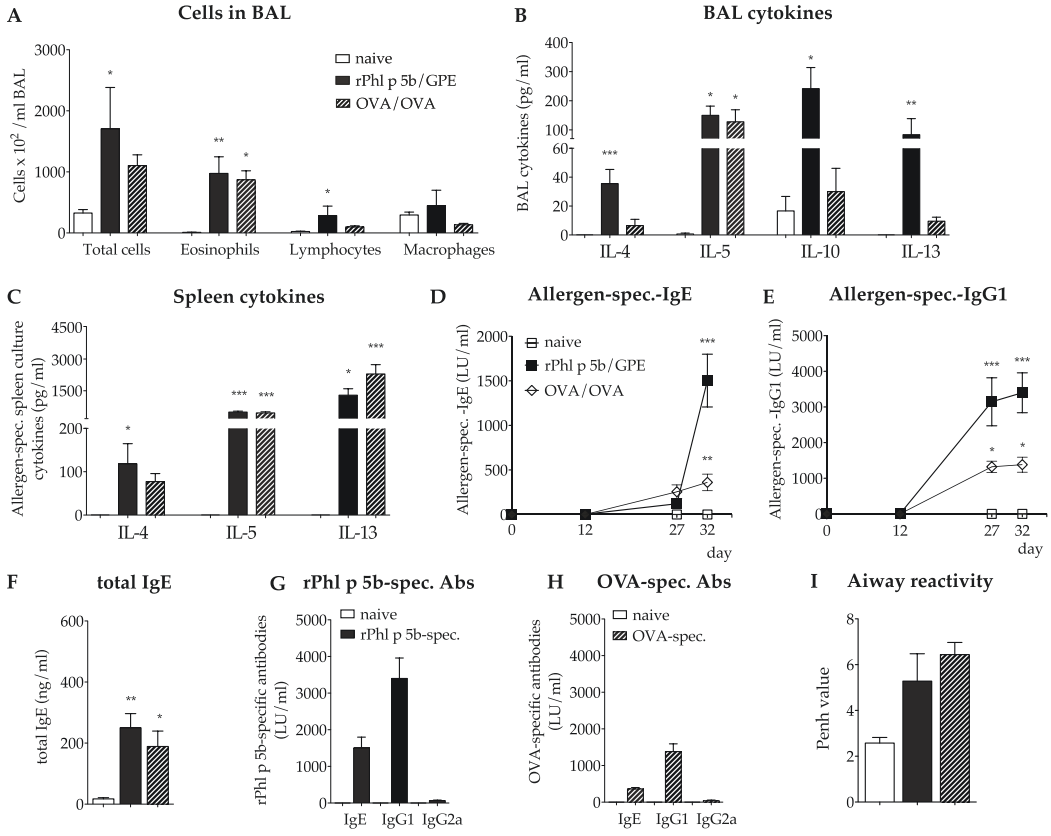

Figure 3.10: Grass pollen-allergen induces stronger inflammatory responses. (A) Number of inflammatory cells detected in the BAL fluid from rPhl p 5b-model and OVA-model. Cytokine levels in (B) the BAL fluid and (C) supernatants from allergen-restimulated spleen cells culture. (D) Allergen-specific IgE and (E) IgG1 antibody kinetics studied before and during sensitization phase, as well as before and after challenges. (F) Total IgE, (G and H) allergen-specific IgE and IgG1 antibody production measured at the day of dissection. (I) Airway hyperreactivity measured at day 31 in response to 50 mg/ml of methacholine. The mean values ±SEM are shown for 5-6 mice per group, representative data of two individual experiments. Compared with naive animals. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ are considered statistically significant.

Cytokine production in the BAL fluid was increased in both models when compared with naive control group. Significantly higher levels of IL-4 were detected in the BAL fluid of animals sensitized and challenged with the grass allergen (42 ±8.9 pg/ml) than mice treated with OVA (6.5 ±4.3 pg/ml). Production of IL-5 was similar in both asthma models, additionally significantly higher levels of IL-10 and IL-13 were measured in the BAL fluid of animals sensitized with rPhl p 5b (Fig. 3.10B). Splenocytes of asthmatic animals from both groups equally proliferated to mitogen restimulation with concavalin A (data not shown) and when restimulated with the respective allergen produced high levels of Th2-associated cytokines (Fig. 3.10C).

Antibody titers indicated no pronounced, increasing production of allergen-specific IgE antibodies after sensitization phase but a dramatic increase of specific IgE production after challenges (Fig. 3.10D). Moreover, the kinetics of the Th2-associated antibody: rPhl p 5b-specific IgG1 showed significantly higher levels in sera of animals from the rPhl p 5b-model than OVA-specific IgG1 in sera of animals from OVA-model (Figure 3.10E, G, H). This may be due to the clinical relevance of grass pollen allergen (discussed in Section 4.2) and/or due to an additional sensitization performed in the rPhl p 5b-model. Assessment of total IgE levels in animals from both asthma models showed significantly higher levels of antibodies in animals from rPhl p 5b-model than in mice from OVA-model (Fig. 3.10F). Allergen-specific IgG2a antibody subclass production was very low in all allergic experimental groups (Fig. 3.10G, H).

In order to assess lung function, AHR to increasing doses of methacholine (MCh) was measured at day 31 on *in vivo* restrained animals by whole body plethysmography. At the dose of 50 mg/ml of MCh animals from both asthma models developed 'longer' breaks in breathing, which was indicated by high Penh (enhanced pause) values. The mean ±SEM of Penh values in the rPhl p 5b-group was 5.3 ±2.9 and in OVA-group 6.4 ±1.2. Naive control mice showed low mean Penh values of 2.6 ±0.6 (Fig. 3.10I). This indicates OVA had a stronger effect in the development of AHR than the grass pollen allergen.

Taken together, these results clearly show that sensitization with a recombinant clinically relevant aeroallergen (rPhl p 5b) and challenges with natural grass pollen extract (GPE) significantly induced all hallmarks of asthma characterized by strong Th2 responses and increased lung function. Moreover, eosinophils and lymphocytes infiltration into the lungs, production of selected Th2-associated cytokines, levels of total IgE, allergen-specific IgE and IgG1 antibodies were induced stronger in the rPhl p 5b-model than the classical OVA-induced asthma model, which has been broadly-used in the allergy research.

3.2.2 Treatment with AvCystatin interferes with the mouse model of allergic asthma induced by the clinically relevant allergen

To study the effect of the recombinant filarial cystatin (rAvCystatin) on allergen-induced sensitization and AHR, the rPhl p 5b-induced murine model was employed, which was established during this work and descried above. For the preventive treatment BALB/c mice were injected i.p. with rPhl p 5b/Alum allergen and were treated i.p. four times in weekly intervals with rAvCystatin and then challenged twice i.n. with GPE (Fig. 3.11). This approach provides rAvCystatin the possibility for interfere with the sensitization phase starting from the first immunization with the allergen. As a control to rAvCystatin, a recombinantly expressed dihydrofolate reductase (rDHFR) protein was applied in the same mode as rAvCystatin. Experimental groups are provided in Table 3.1.

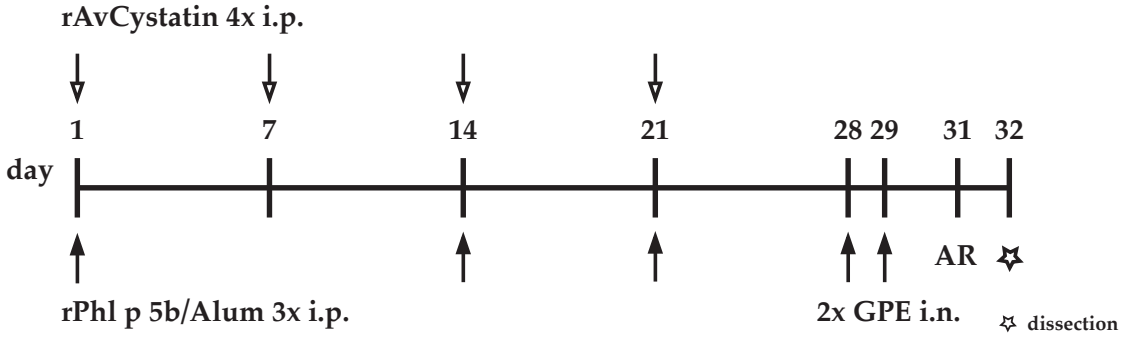

Figure 3.11: Scheme of treatment with rAvCystatin intraperitoneal (i.p.) (Subsection 3.2.2)/ subcutaneous (s.c.) (Subsection 3.2.3) in the rPhl p 5b-induced model of allergic asthma. Animals were treated with rAvCystatin/ control protein rDHFR four times in weekly intervals during the sensitization phase with rPhl p 5b/Alum and challenged two times i.n. with GPE. Naive controls were injected with Alum in PBS and challenged PBS, accordingly. Measurement of airway reactivity (AR) was performed on day 31 and dissection was done one day later.

Table 3.1: Experimental groups described in Subsections 3.2.2, 3.2.3 and 3.3.4.

Group name	Treatment	Sensitization	Challenge
naive	PBS i.p.	PBS i.p.	PBS i.n.
asthmatic controls, rPhl p 5b/GPE	PBS i.p./s.c.	rPhl p 5b i.p.	GPE i.n.
rAvCystatin-treated (also with derivates), rAvCystatin i.p./s.c., rAvCystatin/rPhl p 5b/GPE	rAvCystatin i.p./s.c.	rPhl p 5b i.p.	GPE i.n.
rDHFR-treated, rDHFR/rPhl p 5b/GPE	rDHFR i.p.	rPhl p 5b i.p.	GPE i.n.

AvCystatin reduces airway hyperreactivity and decreases inflammation in the lungs

In vivo development of AHR to increasing doses of MCh was significantly elevated in asthmatic controls when compared with naive controls (p=0.003). Animals treated with rAvCystatin showed significantly decreased AHR when compared with asthmatic controls (p=0.02). There was no change in AHR between a control group treated with the control protein rDHFR (p=0.99) (Fig. 3.12A).

Analysis of the BAL fluid showed that rAvCystatin suppressed airway inflammation as seen by significantly reduced numbers of total cells (p=0.01), numbers of eosinophils (p=0.002) and MCs (p=0.03) when compared with asthmatic controls. Treatment with the control protein rDHFR did not cause significant changes in cell composition of the BAL fluid when compared with asthmatic animals (Fig. 3.12B).

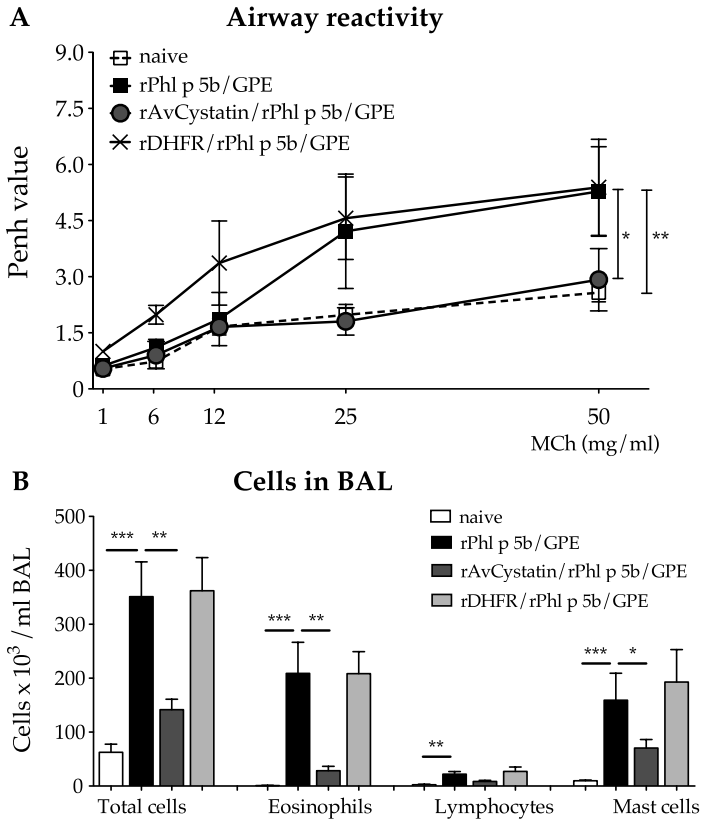

Figure 3.12: Treatment with rAvCystatin suppressed AHR and inflammatory cell infiltration into the lungs. (A) Airway hyperreactivity was measured at day 31 by whole-body plethysmography in response to inhaled methacholine (MCh). AHR is diplayed by Penh (enhanced pause) values. (B) Influx of cells into the BAL fluid. The mean values ±SEM are shown for 5-6 mice per group, representative data of one from three independent experiments. Compared with asthmatic controls. *, p< 0.05; **, p< 0.01; ***, p< 0.001 are considered statistically significant.

In order to evaluate the local effect after treatment with rAvCystatin, staining with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and toluidine blue of the histological lung sections was performed. Analysis of H&E-stained sections showed downregulated local inflammation, reduced eosinophilia around bronchioles and small vessels after treatment with rAvCystatin when compared to control groups (Fig. 3.22C). Moreover, reduced goblet cell hyperplasia was observed in PAS-stained lung sections when compared with asthmatic mice (Fig. 3.22H). As MCs play an important role in the development of allergic asthma, in an immediate reaction as well as in a late phase contributing to lung remodeling and inflammation (see Subsection 1.2.2), the lung tissue was stained with toluidine blue to detect connective tissue MCs residing in lungs. As expected, asthmatic animals showed local infiltration of MCs into the tissue when compared with naive animals (8.5 ±4.6 vs. 5.7 ±2.2 MCs/10 high power fields (hpf)) (Fig. 3.16B). In contrast, rAvCystatin-treated animals exhibited no mastocytosis in the lung tissue (4.5 ±2.1 MCs/10 hpf) (Fig. 3.16C). Lungs of rDHFR-treated animals did not show significant changes when compared with asthmatic controls.

Together, these results indicate that rAvCystatin interferes with allergen-induced inflammatory processes in the lungs.

Altered cytokine production at the site of inflammation after treatment with AvCystatin

Levels of local cytokines were measured *ex vivo* in the BAL fluid. Treatment with rAvCystatin significantly suppressed production of IL-5 (p=0.005) and IL-13 (p=0.02) when compared with asthmatic animals (Fig. 3.13A). However, levels of IL-4 were not significantly changed after rAvCystatin treatment when compared with asthmatic animals (p=0.54). Similarly, IL-10 levels in the BAL fluid showed no statistically significant difference but an increased trend in rAvCystatin-treated animals when compared with allergic controls (p=0.25) (Fig. 3.13A).

Moreover, treatment with rAvCystatin led to a significant suppression of local allergen-specific Th2-type cytokines (IL-4, IL-5 and IL-13; p=0.04, p=0.009, p=0.03, respectively) detected in the supernatants of lung-draining peribronchial lymph node (PBLN) cells restimulated *in vitro* with the allergen rPhl p 5b. Any changes in cytokine levels were not observed after treatment with rDHFR control protein (Fig. 3.13B).

Hence, rAvCystatin downregulates Th2-type cytokines produced at the site of allergic infammation.

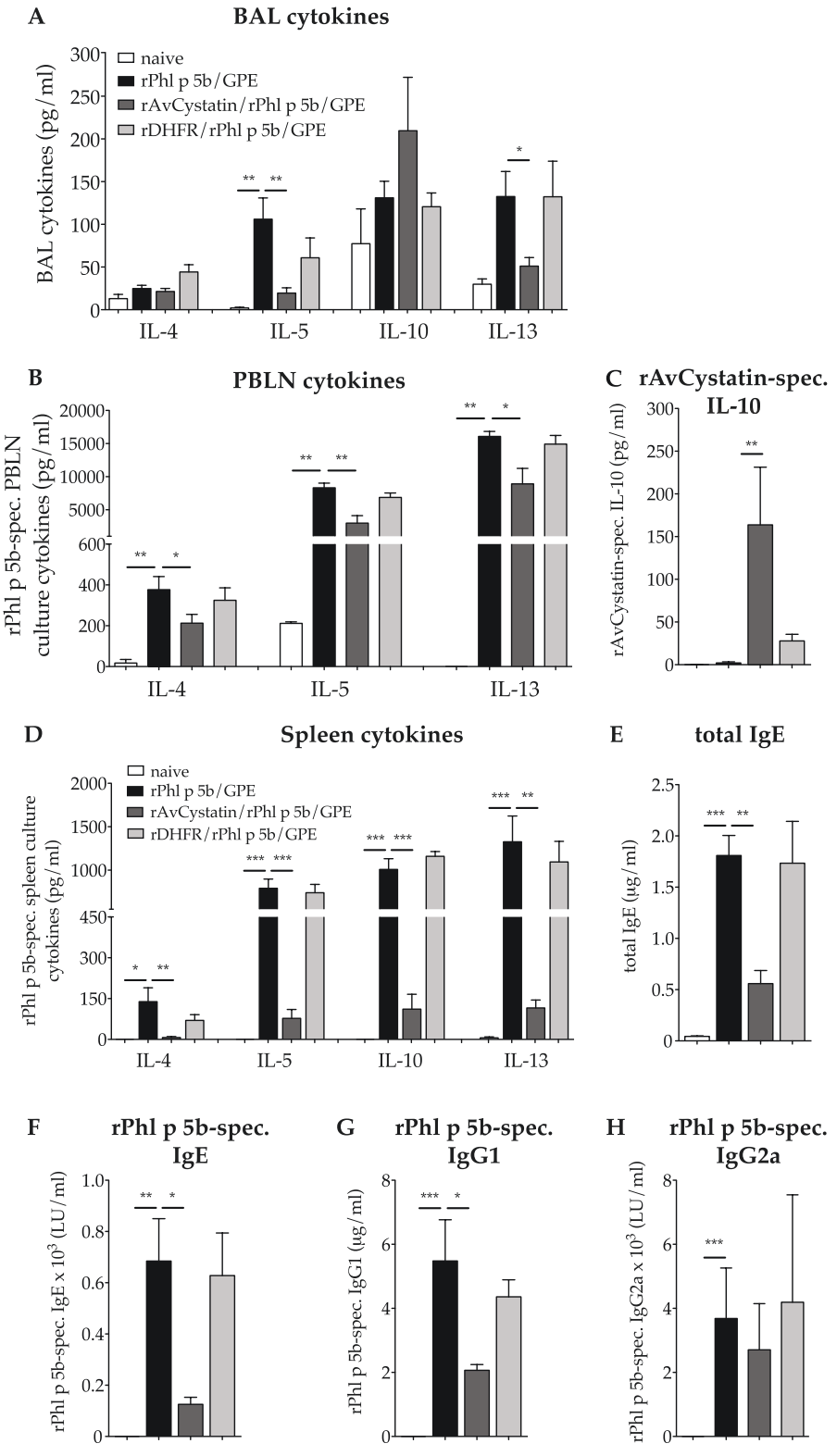

Figure 3.13: Treatment with rAvCystatin interfered with local and systemic allergic responses detected as cytokines (A) in the BAL fluid, (B) in supernatants from PBLN cell cultures and (D) spleen cell cultures restimulated *in vitro* with rPhl p 5b. (C) rAvCystatin-specific IL-10 production by spleen cells. Total IgE (E), allergen-specific IgE (F), IgG1 (G) and IgG2a (H) antibody levels detected in serum. The mean values ±SEM are shown for 5-6 mice per group, representative data from one of three independent experiments. Compared with allergic controls. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ are considered statistically significant.

Downregulated systemic cytokine responses due to AvCystatin treatment

To understand whether AvCystatin also interfered with allergen-specific responses on systemic level the production of Th2-associated cytokines by splenocytes and serum antibody levels were determined. In addition to alleviation of rPhl p 5b-induced allergic asthma in local tissues, AvCystatin significantly downregulated systemic production of grass pollen-specific Th2-type cytokines such as IL-4, IL-5 and IL-13 ($p=0.004$, $p<0.001$ and $p=0.02$, respectively) detected in spleen cells culture supernatants restimulated with rPhl p 5b (Fig. 3.13D). High levels of rPhl p 5b-specific IL-10 were detected in asthmatic animals ($p<0.001$) when compared with naive animals, however the levels were significantly decreased after treatment with rAvCystatin ($p<0.001$) in contrast to treatment of mice with the control protein rDHFR (Fig. 3.13D).

As IL-4 and IL-13 are involved in isotype class switching and/or IgE production, serum antibody levels were analyzed in all groups. Treatment with rAvCystatin significantly decreased production of total IgEs and rPhl p 5b-specific IgEs and IgG1s ($p=0.004$, $p=0.01$ and $p=0.03$, respectively) (Fig. 3.13E-H). No significant changes in rPhl p 5b-specific IgG2a levels were observed when compared with asthmatic controls ($p=0.66$) (Fig. 3.13H). Thus, filarial cystatin shows the systemic effect on downregulation of allergen-specific cytokines and antibodies.

Increased levels of IL-10 and numbers of $CD4^+CD25^+Foxp3^+$ T cells

Although systemic grass pollen-specific IL-10 was reduced by rAvCystatin treatment (Fig. 3.13D), restimulation of splenocytes with rAvCystatin resulted in significant production of IL-10 in animals treated with rAvCystatin during sensitisation ($p=0.01$) (Fig. 3.13C), in line with increased levels of IL-10 in the BAL fluid in the rAvCystatin-treated group (Fig. 3.13A).

Ex vivo responses to rAvCystatin by splenocytes isolated from rAvCystatin-treated animals did not lead to a significant increase in any other cytokines examined (IL-12p40, IFN-γ, IL-4, IL-5, IL-13 or IL-6, data not shown). Thus, our data are in line with previous findings that filarial cystatin specifically induces the expression of IL-10 in the murine system.

Regulatory cells also play an important role in downmodulation of allergy and asthma. Isolated splenocytes were analyzed for the *ex vivo* expression of regulatory T cell markers including CD25 and Foxp3. Elevated total numbers of $CD4^+CD25^+Foxp3^+$ T cells were detected in spleens from rAvCystatin-treated animals when compared with asthmatic controls ($p=0.02$) (Fig. 3.14B).

These results indicate that rAvCystatin interfered with a murine model of allergic asthma induced by a clinically relevant allergen and at the same time induced potentially regulatory mechanisms such as IL-10 and $CD4^+CD25^+Foxp3^+$ T cells.

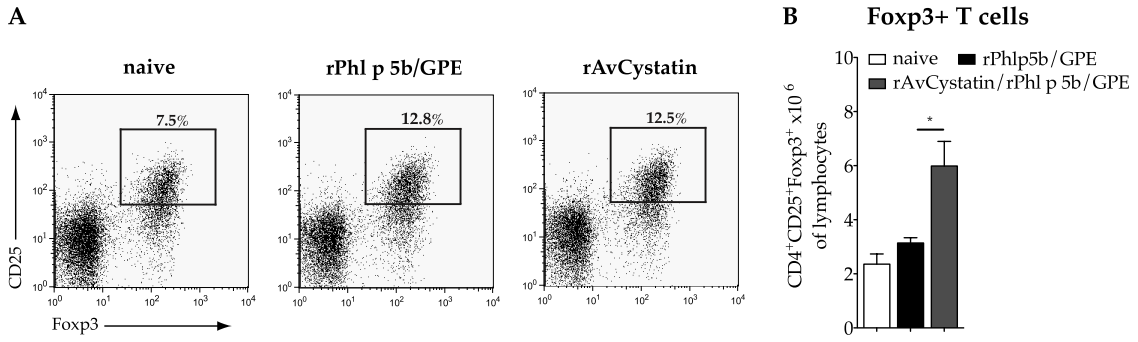

Figure 3.14: Frequencies of $CD4^+CD25^+Foxp3^+$ T cells in the spleen after treatment with rAvCystatin. (A) FACS plots of $CD4^+$ T cells from one representative animal per group. (B) Total numbers of $CD4^+CD25^+Foxp3^+$ T cells in the spleen. The mean values ±SEM from one experiment with 5-6 mice per group. Compared with allergic controls. *, $p < 0.05$ is considered statistically significant.

3.2.3 Routes of AvCystatin administration: intraperitoneal versus subcutaneous

In order to explore the efficacy of different routes of application, rAvCystatin was applied subcutaneously (s.c.) during the sensitization phase with rPhl p 5b as illustrated in Fig. 3.11. In this approach animals from s.c. rAvCystatin-treated group were analyzed together with i.p. rAvCystatin-treated animals and characteristic parameters of allergic airway hyperreactivity and inflammation were measured. Experimental groups are in Table 3.1.

Subcutaneous application of AvCystatin does not downmodulate allergic asthma

The application of rAvCystatin i.p. as presented before led to suppression of all main hallmarks of allergic asthma, however application of rAvCystatin s.c. did not show such effect. The lung function of mice measured by whole-body plethysmography in response to inhaled increasing doses of MCh of rAvCystatin s.c.-treated animals was not significantly changed when compared with allergic controls (p=0.9) (Fig. 3.15A).

In line with this, rAvCystatin s.c.-treated animals did not show decrease of total cell or lymphocyte numbers in the BAL fluid. However, s.c.-treated animals showed significantly increased numbers of eosinophils (p=0.04) and strikingly significantly decreased MCs numbers (p=0.03) locally when compared with asthmatic animals (Fig. 3.15B).

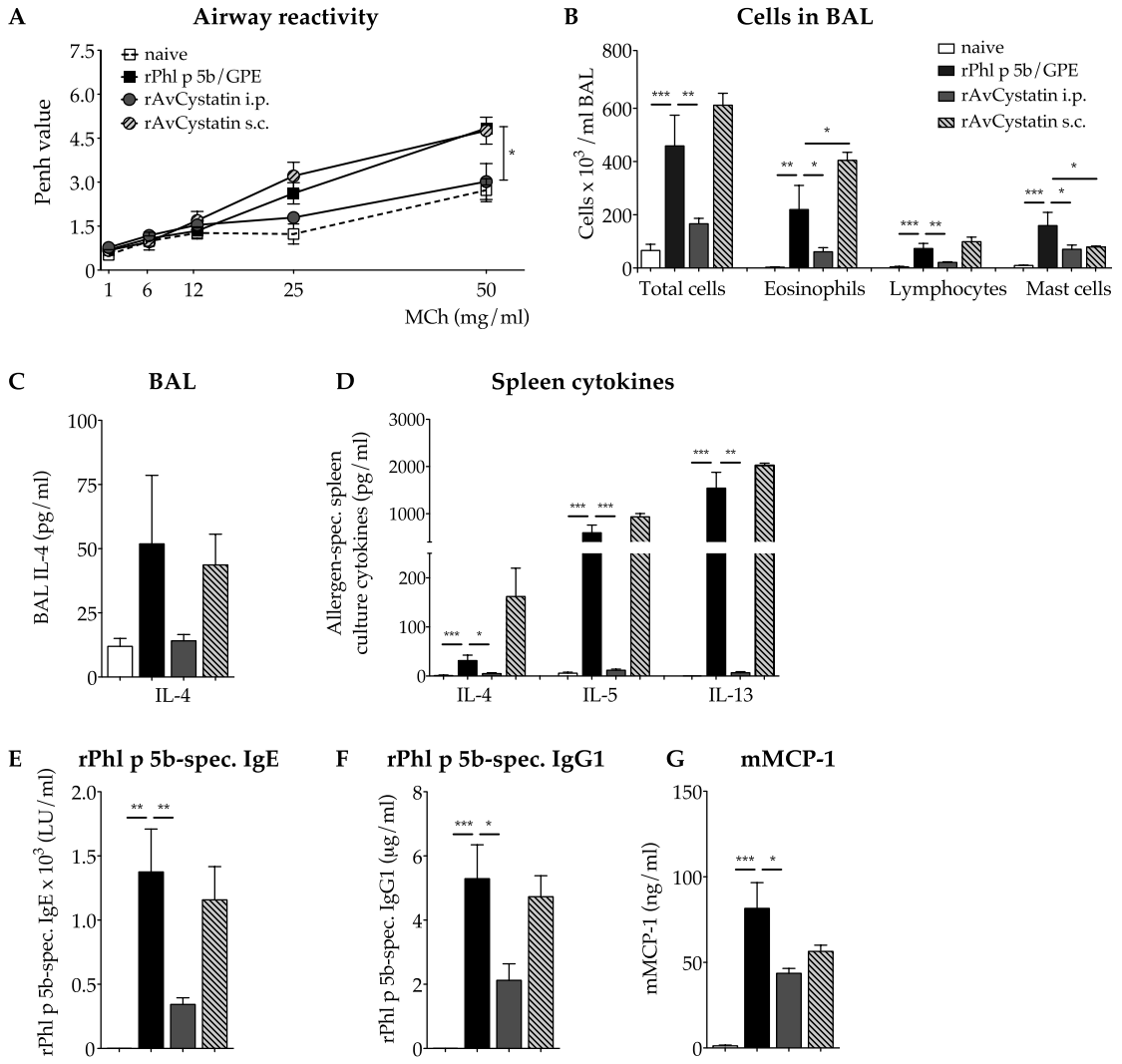

Figure 3.15: Subcutaneous treatment with rAvCystatin did not suppress Th2-type allergic responses. (A) Airway hyperreactivity was measured by whole-body plethysmography at day 31 in response to inhaled methacholine (MCh). (B) Cell numbers in the BAL fluid. (C) IL-4 levels in the BAL fluid. (D) Cytokines detected in supernatants of spleen cells in response to the allergen. (E) and (F) Allergen-specific antibody levels detected in serum. (G) Mouse mast cell protease-1 (mMCP-1) levels in serum. The mean values ±SEM of one experiment with 4-6 animals per group. Compared with asthmatic controls. *, p< 0.05; **, p< 0.01; ***, p<0.001 are considered statistically significant.

Analysis of cytokines in the BAL fluid of animals treated with rAvCystatin s.c. showed unaltered production of IL-4 (p=0.74) (Fig. 3.15C), IL-5 and IL-13 (data now shown) when compared with asthmatic animals. No difference was observed in cytokine levels in supernatants from spleen cells cultured with the allergen rPhl p 5b (Fig. 3.15D). Finally, elevated levels of allergen-specific IgE and IgG1 were observed in s.c.-treated animals (p=0.62 and p=0.63, respectively) (Fig. 3.15E, F).

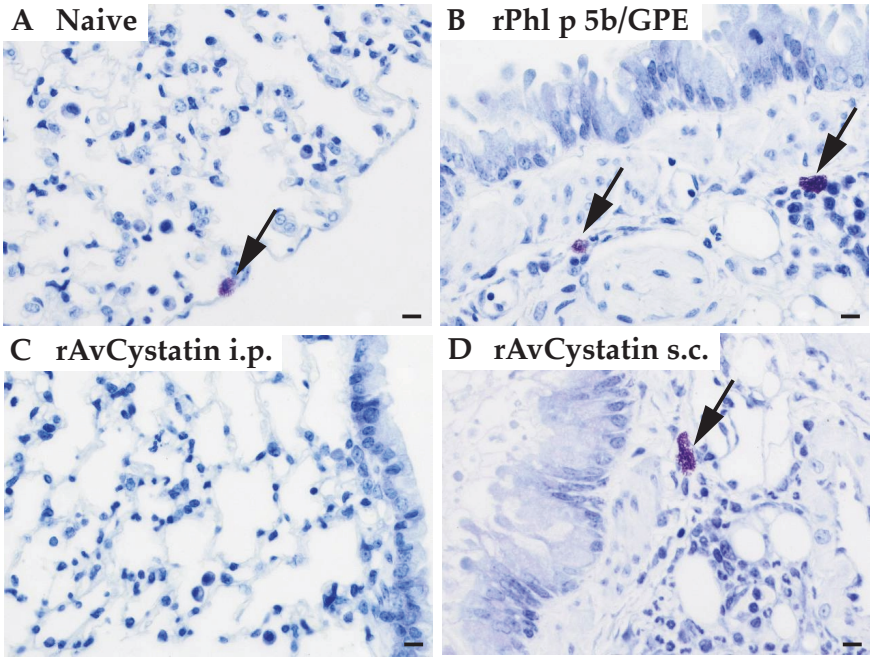

Figure 3.16: Application of rAvCystatin i.p. and s.c. reduced numbers of mast cells (MCs) in the lungs. Representative lung sections stained with toluidine blue for resident connective tissue MCs (black arrows). Quantification of the data in the text. Naive: naive control, rPhl p 5b/GPE: asthmatic; rAvCystatin i.p.: rAvCystatin-treated i.p.; rAvCystatin s.c.: rAvCystatin-treated s.c. animals. Original magnification: x400, right side scale bars = 20 μm.

In order to explore on the level of activation and degranulation of MCs, the serum was analyzed for mMCP-1. Treatment with rAvCystatin resulted in decreased mMCP-1 levels, which were significantly reduced after i.p.-treatment (p=0.018 and p=0.13, respectively) (Fig. 3.15G). Moreover, rAvCystatin i.p. (4 ±2.8 MCs/10 hpf) and to lesser extend rAvCystatin applied s.c. (7 ±2.1 MCs/10 hpf), decreased infiltration of MCs in the lungs as indicated by toluidine blue staining of lung sections when compared with asthmatic animals (10.7 ±1.1 MCs/10 hpf) (Fig. 3.16A-D).

The immunomodulatory effect was observed only when of rAvCystatin was applied intraperitoneally but not subcutaneously. This indicates that rAvCystatin downregulates allergic disease depending on the specific way of its administration.

3.3 Structural and functional aspects of AvCystatin in the immunomodulatory effect

In order to explore, whether main features of cystatins play a role in the immunomodulatory effect of rAvCystatin, production and analysis of three forms of the molecule was performed: (1) a mutated form, not active as protease inhibitor; (2) truncated, mainly monomeric version; (3) demethylated molecule, designed to lack a posttranslational modification (PTM) (Fig. 3.17B-D).

Figure 3.17: Scheme of the four derivates of AvCystatin molecule. (A) recombinant unmodified rAvCystatin; (B) mutated: rAvCystatin$_{mut}$; (C) truncated: rAvCystatin$_{tr}$ and (D) demethlyted: rAvCystatin$_{dm}$. 'VLVRC' (grey): as part of the N-terminal extension five amino acids including one of the three cysteines 'C'; 'LLGG', 'QVVAG' and 'SW': three conserved motifs involved in active centers constituting cysteine protease inhibitor activity (red). 'C': conserved cysteine residues forming disulphide bonds (between the second and third conserved domain); 'K': lysine, site of the posttranslational modification (methylation). Information on specific changes of AvCystatin molecules are described in the text.

3.3.1 Protease inhibitor activity: mutated AvCystatin

In order to study an influence of rAvCystatin's protease inhibitor activity mediated by three highly conserved motifs involved in active centers, a mutated derivate of rAvCystatin (rAvCystatin$_{mut}$) was produced.

The work was done in our group (J. Russ and T. Buhrke, unpublished) and is described in detail in Section 6.1. Recombinant AvCystatin$_{mut}$ was obtained by site-directed mutagenesis of each from three conserved domains to, first of all disrupt the protease inhibition function and at the same time to preserve the overall conformational structure of the molecule. Thus, two leucines and two glycines (LLGG) from N-terminal domain were exchanged to serine and three alanines (SAAA), from

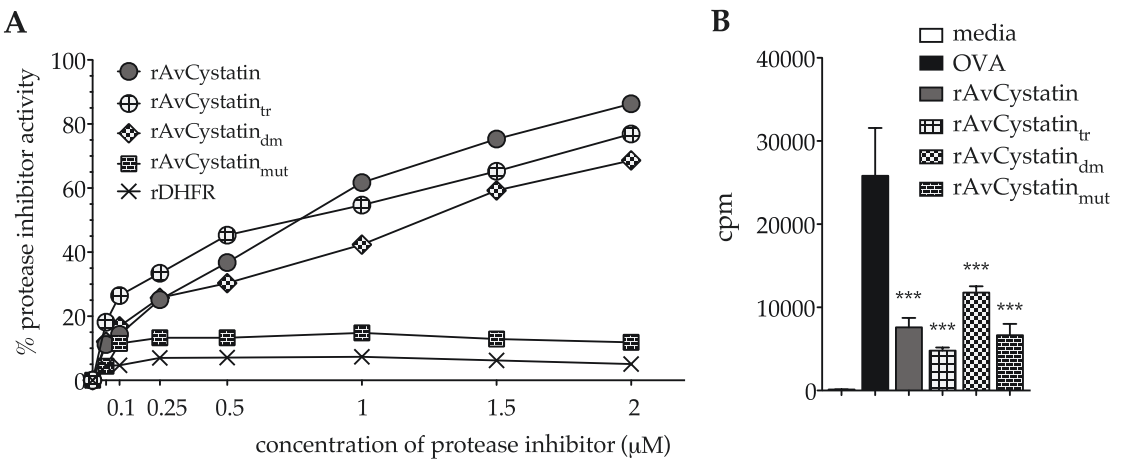

Figure 3.18: Protease inhibitor activity and immunosuppressive effect of rAvCystatin and derivates. (A) Protease inhibitor activity to papain cleavage of increasing concentrations of recombinant AvCystatin and three derivates, and the control protein rDHFR. (B) *in vitro* ^{3}H-thymidine incorporation assay by proliferating splenocytes from DO10.11 mice stimulated with OVA, rAvCystatin and derivates. Stimulations: media: media control, OVA: ovalbumine, rAvCystatin: recombinant cystatin plus OVA, rAvCystatin$_{tr}$: truncated cystatin plus OVA, rAvCystatin$_{dm}$: demethylated cystatin plus OVA, rAvCystatin$_{mut}$: mutated cystatin plus OVA; cpm: counts per minute. Compared with OVA stimulation. ***, $p<0.001$ is considered statistically significant.

the second motif one glutamine and two valines (QVV) were mutated to serine, alanine and serine (SAS), tryptophan (W) was changed into alanine (A) in the C-terminal conserved domain. All other parts of the primary structure stayed unaltered (Fig. 3.17B).

Further, the protease inhibitor activity to papain cleavage of *E. coli*-expressed and purified rAvCystatin$_{mut}$ was evaluated in the inhibitor activity assay as described in Hartmann et al. (1997). Mutated cystatin rAvCystatin$_{mut}$ was not inhibitory and showed only a baseline 11% of protease inhibitor activity, the same as the control protein rDHFR. In contrast, unmodified rAvCystatin and other derivates (presented below) showed strong protease inhibitor activity proportionally increasing to raising concentrations of the inhibitor and reaching 80% of inhibitor activity at 2 μM cystatin concentration (Fig. 3.18A).

In order to evaluate the suppressive capacity of rAvCystatin$_{mut}$, a ^{3}H-thymidine incorporation assay of proliferating splenocytes stimulated with OVA and with other rAvCystatin's derivates was applied *in vitro*. Mutated rAvCystatin$_{mut}$ as well as unmodified rAvCystatin suppressed OVA-induced proliferation of splenocytes from DO10.11 mice (Fig. 3.18B).

Data suggest that protease inhibitor activity does not confer immunomodulatory property of rAvCystatin *in vitro*.

3.3.2 Monomers and dimers formation: truncated AvCystatin

Naturally cystatins appear as monomers, however under special conditions they are able to form dimers or oligomers (discussed in Section 4.3). Coaggregations of cystatin were observed in preparations of the recombinant protein and cDNA sequence analysis revealed that rAvCystatin carries an additional N-terminal cysteine residue that could serve as a source of newly formed disulfide bridge between another cystatin molecule and in consequence leading to aggregation of the protein. In order to study this phenomena, the five N-terminal amino acids including extra cysteine (VLVRC) were truncated (Buhrke, not published), modified cDNA was cloned, *E. coli*-expressed and purified rAvCystatin$_{tr}$ was obtained.

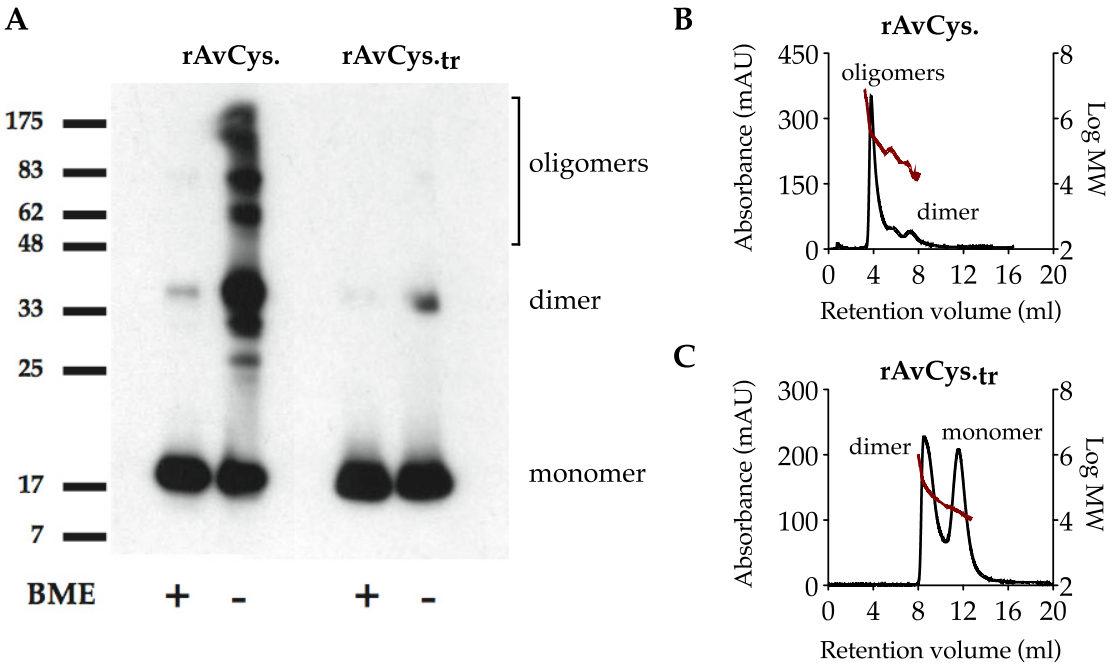

Figure 3.19: Profile of unmodified rAvCystatin and truncated rAvCystatin$_{tr}$. (A) Western blot analysis with (+) and without (-) the reducing agent β-mercaptoethanol (BME). rAvCystatin includes bands at around 18 kDa corresponding to monomers, 36 kDa to dimers and heavier than 48 kDa - oligomers. rAvCystatin$_{tr}$ shows two major bands at 18 kDa and 36 kDa. (B, C) Profile of examined proteins in solution by analytical gel filtration and right angle static light scattering (RALS). (B) rAvCystatin is predominnalty oligomeric with small proportion of identified dimers and monomers having a predicted monomer mass of 18 kDa. (C) rAvCystatin$_{tr}$ contains a monomer:dimer equilibrium. Absorbance was measured at 280 nm; MW: molecular weight.

The capability of recombinant AvCystatin to dimerize and/or oligomerize was checked by Western blot analysis and right angle static light scattering (RALS). Western blot showed that unmodified rAvCystatin appeared as a mixture of mono-

mers, dimers and oligomers. The truncated derivate lacking the first five N-terminal amino acids (rAvCystatin$_{tr}$) was detected as a majority of monomers and to lesser extend dimers, as shown in Western blot under non-reducing conditions (Fig. 3.19A).

Results from this immunological method were confirmed by analytical gel filtration analysis and by RALS. The RALS-analyzed samples had a protein concentration of 3-5 mg/ml. Thus, monomers were separated from dimers and oligomers. Next, molecular weights (MW) of appearing peak populations in the solution were estimated and the profile of examined proteins was graphed. After RALS rAvCystatin was found to be predominantly oligomeric with a recognized molecular weight of 186 kDa and greater. Unless in Western blot analysis, smaller proportions of dimers and monomers of a predicted mass of 36 kDa and 18 kDa, respectively, were identified (Fig. 3.19B). Absorbance trace from a column loaded with rAvCystatin$_{tr}$ showed dimer and monomer peaks only with a predicted monomer mass of 18 kDa, which confirms results from Western blot analysis (Fig. 3.19C).

These data provide an evidence of heavy oligomerization of unmodified rAvCystatin. On the other hand rAvCystatin$_{tr}$ lacking an extra N-terminal cysteine is monomeric in the solution and has the capability to assemble into dimers at high protein concentrations.

3.3.3 Posttranslational modification: demethylated AvCystatin

Many proteins undergo various posttranslational modifications, which can play important functional roles. In order to analyze a newly identified methylation moiety of rAvCystatin (identified in a cooperation with AG Schmieder; T. Helmbrecht, personal communication), removal of a methylation at lysine (K) via site-directed DNA mutagenesis (p.K137L) was done in frame of this work.

```
                301                             330                             360
                 |                               |                               |
rAvCys           GTTGCTCAATCAGAATGCAAAAAAAGTTCAGGCGAGGAAGTTAATCTGAAAACATGTAAA
rAvCysdm         GTTGCTCAATCAGAATGCAAAAAAAGTTCAGGCGAGGAAGTTAATCTGAAAACATGTAAA
                 ************************************************************

                361                             390                 409         420
                 |                               |                   |           |
rAvCys           AGATTGGAAGGACATCCGGATCAGATTATCACGTTGGAGGCATGGGAGAAATCATGGGAA
rAvCysdm         AGATTGGAAGGACATCCGGATCAGATTATCACGTTGGAGGCATGGGAGCTGTCATGGGAA
                 ************************************************    *********

                421                             450                 471
                 |                               |                   |
rAvCys           AATTTTTTGCAAGTCAAAATTCTGGAAAAAAAAGAAGTACTCTCATCAGTG
rAvCysdm         AATTTTTTGCAAGTCAAAATTCTGGAAAAAAAAGAAGTACTCTCATCAGTG
                 ***************************************************
```

Figure 3.20: Alignment of rAvCystatin and rAvCystatin$_{dm}$ cDNA sequences (part). In red marked mutation site c.409_411AAA>CTG, which results in p.K137L. Consensus: identical residues are indicated by (*).

A successful nucleotide mutation in DNA sequence of rAvCystatin in pET28b vector was confirmed by DNA sequencing (Fig. 3.20). Indeed, the introduced mutation at c.409_411AAA>CTG was identified, which in consequence replaced lysine (K) by leucine (L), p.K137L (Fig. 3.17D).

In frame of this work, the *E. coli*-recombinantly expressed demethylated rAvCystatin (rAvCystatin$_{dm}$) was purified and checked in a functional *in vitro* assay. The suppressive function of rAvCystatin$_{dm}$ was confirmed by the ^{3}H-thymidine incorporation assay of *in vitro* proliferating splenocytes stimulated with OVA and rAvCystatin's derivates (Fig. 3.18B). Demethylated rAvCystatin$_{dm}$, equally as unmodified rAvCystatin, suppressed OVA-induced proliferation of splenocytes from DO10.11 mice as measured by the incorporated ^{3}H-thymidine and displayed as counts per minute (cpm) (Fig. 3.18B).

These data suggest the identified PTM is not responsible for rAvCystatin's immunosuppressive capacity *in vitro*. Yet, its *in vivo* capacity could be addressed in future studies.

3.3.4 Functional analysis of AvCystatin's derivates in a murine model with a clinically relevant allergen

To study the functional effect *in vivo* of recombinant AvCystatin's derivates, rAvCystatin$_{tr}$, rAvCystatin$_{mut}$ and rAvCystatin (as a positive control) were applied in the model of allergic airway hyperreactivity and inflammation induced by grass pollen allergen as shown in Fig. 3.11. Experimental groups are in Table 3.1.

The suppressive capacity of cystatin is independent of oligomerization and protease inhibitor activity

Analysis of the BAL fluid and histology of lungs revealed that rAvCystatin derivates applied during sensitiation with rPhl p 5b suppressed local inflammation. All forms of cystatin (unmodified rAvCystatin, rAvCystatin$_{tr}$, rAvCystatin$_{mut}$) significantly reduced numbers of total cells ($p=0.01$, $p=0.02$, $p=0.01$, respectively) and eosinophils ($p=0.0008$, $p=0.0002$, $p=0.0009$, respectively) in the BAL fluid when compared with asthmatic animals (Fig. 3.21A).

Staining of lung tissues with H&E and PAS confirmed that treatment with any of the rAvCystatin derivate interfered with allergen sensitization and challenge resulting in inhibition of eosinophils recruitment into the lung tissue, reduction of small vessel inflammation and decreased goblet cell hyperplasia in the lungs (Fig. 3.22).

Additionally, all derivates of the rAvCystatin had downmodulatory systemic effects. Production of IL-4, IL-5 and IL-13 by spleen cells in response to rPhl p 5b was significantly decreased after treatment with rAvCystatin$_{tr}$, rAvCystatin$_{mut}$ and rAvCystatin when compared with asthmatic animals (Fig. 3.21B). Treatment with rAvCystatin$_{tr}$ and rAvCystatin$_{mut}$ during sensitization significantly reduced rPhl p 5b-specific IgE (p=0.02, p=0.02) and IgG1 (p=0.03, p=0.02) production, considered characteristic for Th2-cell responses (Fig. 3.21C, D).

In summary, the effect of treatment with AvCystatin on local and systemic inflammation in the murine model of allergic asthma induced by the clinically relevant allergen was independent of aggregations: dimerization and/or oligomerization via disulfide bonds or of the biological function as cysteine protease inhibitor. In time frame of this work, it was not possible to test whether the immunomodulatory effect *in vivo* depends on the identified PTM - a methylation residue. This aspect may be addressed in future studies *in vivo* utilizing produced demethylated rAvCystatin's molecule.

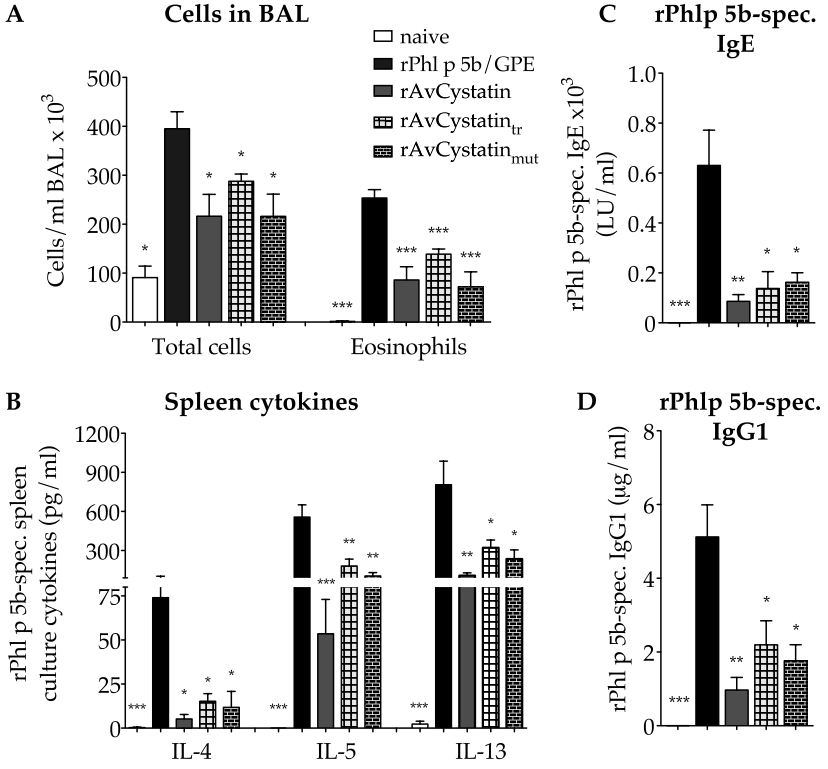

Figure 3.21: Local and systemic responses are suppressed after treatment with rAvCystatin and derivates. (A) Total cells and eosinophils numbers in the BAL fluid. (B) Cytokines detected in supernatants of spleen cells responding to the rPhl p 5b allergen *in vitro*. Allergen-specific IgE (C) and IgG1 (D) antibody levels detected in serum. The mean values ±SEM of two independent experiments with 5-6 animals per group. Compared with asthmatic controls. *, p< 0.05; **, p< 0.01; ***, p<0.001 are considered statistically significant.

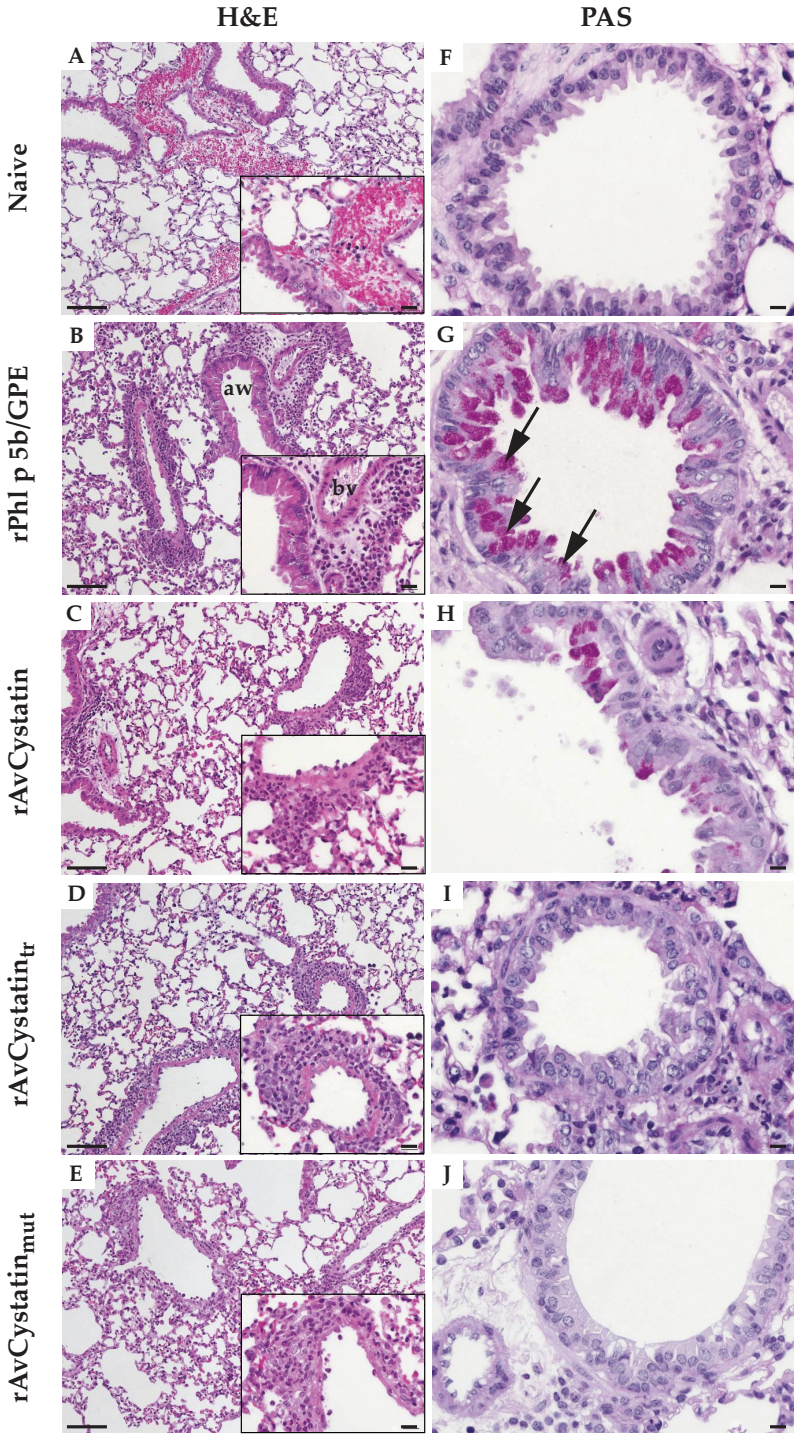

Figure 3.22: Application of rAvCystatin and derivates suppressed inflammatory changes in the lungs. Representative lung sections stained with H&E (A-E) for detection of eosinophils. In blue: nuclei of cells, in red eosinophilic structures. (F-J) Staining with PAS for analysis of goblet cells. Black arrows indicate goblet cells producing mucus. Naive: naive control, rPhl p 5b/GPE: asthmatic; rAvCystatin: rAvCystatin- or derivate-treated animals; aw: airways; bv: blood vessel. Original magnification: H&E x100 (inset x400); PAS x400; left side scale bars = 100 μm, right side scale bars = 20 μm.

3.4 Allergen-specific immunotherapy and adjuvant capacity of AvCystatin

Currently the only successful and effective treatment available for allergy is an allergen-specific immunotherapy (SIT). However, there is a need for improving the efficacy of this treatment as well as reducing the incidence and severity of adverse reactions. Thus, efforts to tackle this matter include the use of modified allergens, alternative routes of administration and novel adjuvants.

In order to explore the capacity of filarial cystatin as an adjuvant in SIT, a mouse model of OVA-specific immunotherapy was established in frame of this dissertation (in a cooperation with AG Hamelmann) and rAvCystatin, as an adjuvant, was applied together with the model allergen OVA.

3.4.1 Establishment of a model: OVA-specific immunotherapy suppresses airway manifestations in asthmatic mice

Two i.p. sensitizations with OVA/Alum and three aerosol challenges with OVA in PBS were applied to BALB/c mice. The allergen-specific immunotherapy (SIT) treatment was performed s.c. with high dose (SIT high) or lower dose (SIT low) of OVA between the sensitization and the challenge phase. One day before analysis, airway reactivity (AR) was measured *in vivo* (Fig. 3.23). Experimental groups are provided in Table 3.2.

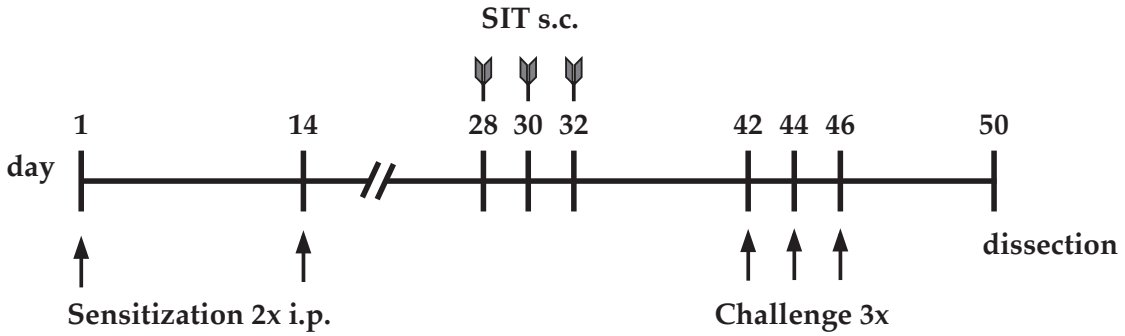

Figure 3.23: Scheme of the OVA-induced immunotherapy model of allergic asthma. Sensitization 2x i.p. with OVA/Alum in two-week intervals, SIT s.c.: 3x OVA allergen-specific s.c. treatment with either SIT high or SIT low, followed by 3x aerosol challenge with OVA.

Table 3.2: Experimental groups described in Section 3.4

Group name	Sensitization	SIT/Therapy	Challenge
naive	PBS i.p.	PBS s.c.	OVA aerosol
asthmatic, OVA/OVA	OVA i.p.	PBS s.c.	OVA aerosol
SIT high	OVA i.p.	OVA 1 mg s.c. (SIT high)	OVA aerosol
SIT low	OVA i.p.	OVA 100 μg s.c. (SIT low)	OVA aerosol
rAvCystatin + OVA s.c.	OVA i.p.	rAvCystatin mixed, injected s.c. together with SIT low	OVA aerosol
rAvCystatin s.c. & OVA s.c.	OVA i.p.	rAvCystatin s.c. injected separately from SIT low	OVA aerosol
rAvCystatin i.p. & OVA s.c.	OVA i.p.	rAvCystatin injected i.p. separately from SIT low	OVA aerosol
rAvCystatin i.p.	OVA i.p.	rAvCystatin i.p.	OVA aerosol

In the OVA-induced immunotherapy model of allergic asthma, SIT treatment was performed three times every second day starting at day 28. High and low doses of the allergen - OVA were applied in s.c. injections.

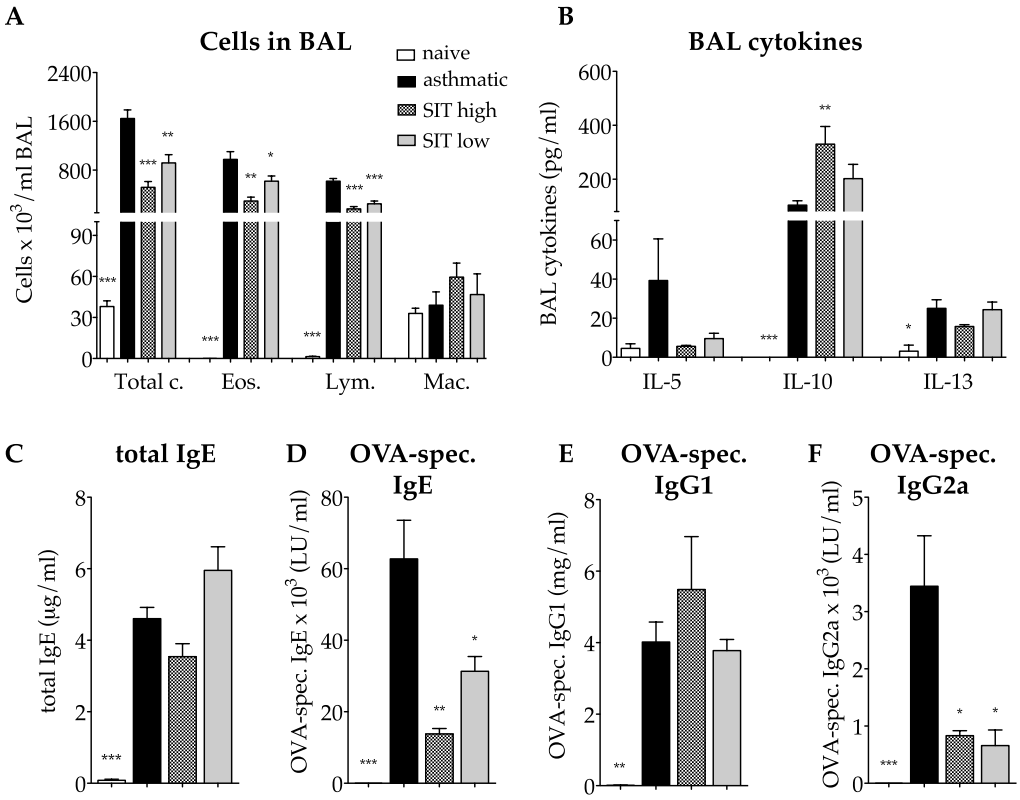

Figure 3.24: Local and systemic responses are suppressed after SIT treatment in the OVA-induced immunotherapy model of allergic asthma. (A) Total cells, eosinophils, lymphocytes and macrophages numbers in the BAL fluid. (B) Cytokines detected in the BAL fluid. (C) Total IgE, (D) OVA-specific IgE, (E) IgG1 and (F) IgG2a levels detected in serum. The mean values ±SEM of one from three independent experiments, 4-5 animals per group. Compared with asthmatic controls. *, $p< 0.05$; **, $p< 0.01$; ***, $p<0.001$ are considered statistically significant.

To evaluate the effect of repeated s.c. administration of the allergen, local and systemic level of inflammatory responses was assessed. Significantly reduced numbers of total cells, eosinophils and lymphocytes were observed in the BAL fluid after SIT high (p=0.0004, p=0.003 and p=0.0001, respectively) and SIT low (p=0.0059, p=0.04 and p=0.0003, respectively) treatment. Numbers of macrophages in the BAL fluid were not significantly changed after SIT high (p=0.19) or after SIT low (p=0.67) application when compared with asthmatic animals (Fig. 3.24A).

Analysis of cytokines in the BAL fluid indicated that SIT high and SIT low treatment decreased, however not significantly, IL-5 and IL-13 levels (p=0.11, p=0.9, respectively). On the other hand, SIT high significantly increased production of IL-10, an important regulatory cytokine in asthma treatment, but not SIT low (p=0.007, p=0.11, respectively) (Fig. 3.24B).

Allergen-specific immunotherapy had also a systemic effect in this allergy model. Levels of total IgEs were decreased after SIT high treatment and surprisingly increased after SIT low, however changes were not statistically significant (p=0.06, p=0.1, respectively) (Fig. 3.24C). High and low doses of SIT significantly decreased allergen-specific IgE and IgG2a antibody production (SIT high: p=0.005, p=0.03 and SIT low: p=0.02, p=0.03, respectively) (Fig. 3.24D, F). Only SIT high showed trend towards increased production of allergen-specific IgG1 antibodies (p=0.34), levels of those stayed unaltered after SIT low application (p=0.71) (Fig. 3.24E).

The OVA-induced immunotherapy murine model of allergic asthma and application of the allergen as therapy were successfully established. SIT high treatment had more beneficial effects than application of SIT low.

3.4.2 Adjuvant capacity and routes of AvCystatin administration in the immunotherapy model

As the SIT high showed already very strong suppressive effect on allergic asthma, treatment with SIT low seemed possible to be further improved. Therefore, SIT low therapy was chosen to be applied together with a potential adjuvant, rAvCystatin.

Subsequently to test an adjuvant capacity of rAvCystatin, the molecule was mixed with OVA and applied all together s.c. (rAvCystatin + OVA s.c.) as a therapy in the first approach. In the second and third approach, it was administered in two separate, either s.c. or i.p., injections at the same time with SIT low (rAvCystatin s.c. & OVA s.c. or rAvCystatin i.p. & OVA s.c.) (Table 3.2 and Fig. 3.25).

Administration of rAvCystatin as an adjuvant in SIT low did not improve AHR. The function of asthmatic lungs was even exacerbated after rAvCystatin-adjuvant to SIT therapy (Fig. 3.26A).

Additional suppressive capacity of rAvCystatin as an adjuvant was not observed locally after examination of the BAL fluid for total cell, eosinophils or lymphocytes influx when compared with SIT low group. Surprisingly, a marked increase in numbers of total cells and eosinophils into the site of inflammation was observed after administration of rAvCystatin i.p. & OVA s.c. (p=0.03, p=0.009, respectively) (Fig. 3.26B). This suggests i.p. application of rAvCystatin as an adjuvant in allergen-specific immunotherapy aggravates local inflammation although SIT low was applied.

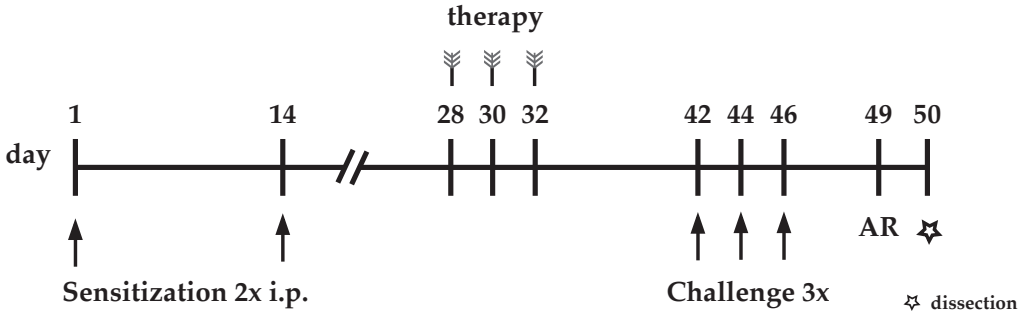

Figure 3.25: Scheme of allergen-specific immunotherapy treatment with AvCystatin as an adjuvant in the OVA-induced immunotherapy model of allergic asthma. Sensitization 2x i.p. with OVA/Alum in two-week intervals, therapy (four separate groups): SIT low s.c.; rAvCys + OVA s.c.; rAvCys s.c. & OVA s.c.; rAvCys i.p. & OVA s.c., followed by 3x aerosol challenge with OVA. Airway reactivity (AR) analysis was performed on day 49 and dissection was done one day later.

Analysis of systemic responses did not indicate further improvement. In addition, rAvCystatin when applied s.c. as an adjuvant to SIT low (mixed or as a separate injection) significantly magnified production of IL-4 (p=0.003, p=0.0003, respectively) and IL-5 (p=0.02, p=0.04, respectively) by spleen cells restimulated with OVA *in vitro*. Levels of IL-10 (p=0.26, p=0.43) and IL-13 (p=0.11, p=0.59) were unchanged in those two groups. On the other hand a significant decrease of OVA-specific IL-4 and IL-10 production was observed after administration of rAvCystatin i.p. & OVA s.c. (Fig. 3.26C, D). Allergen-specific IgE production was unaltered after adjuvant rAvCystatin application. However, a significantly increased OVA-specific IgG1 was determined in sera from rAvCystatin s.c. + OVA s.c.- and rAvCystatin i.p. & OVA s.c.-treated animals when compared with SIT low-treated animals (p=0.0001, p=0.0004, respectively). No significant increase in OVA-specific IgG2a levels was observed (Fig. 3.26E-G). Thus, data do not show conclusive results.

Application of rAvCystatin did not show beneficial adjuvant capacity to SIT low. Moreover, in some instances rAvCystatin was aggravating hallmarks of asthma when applied together with SIT treatment.

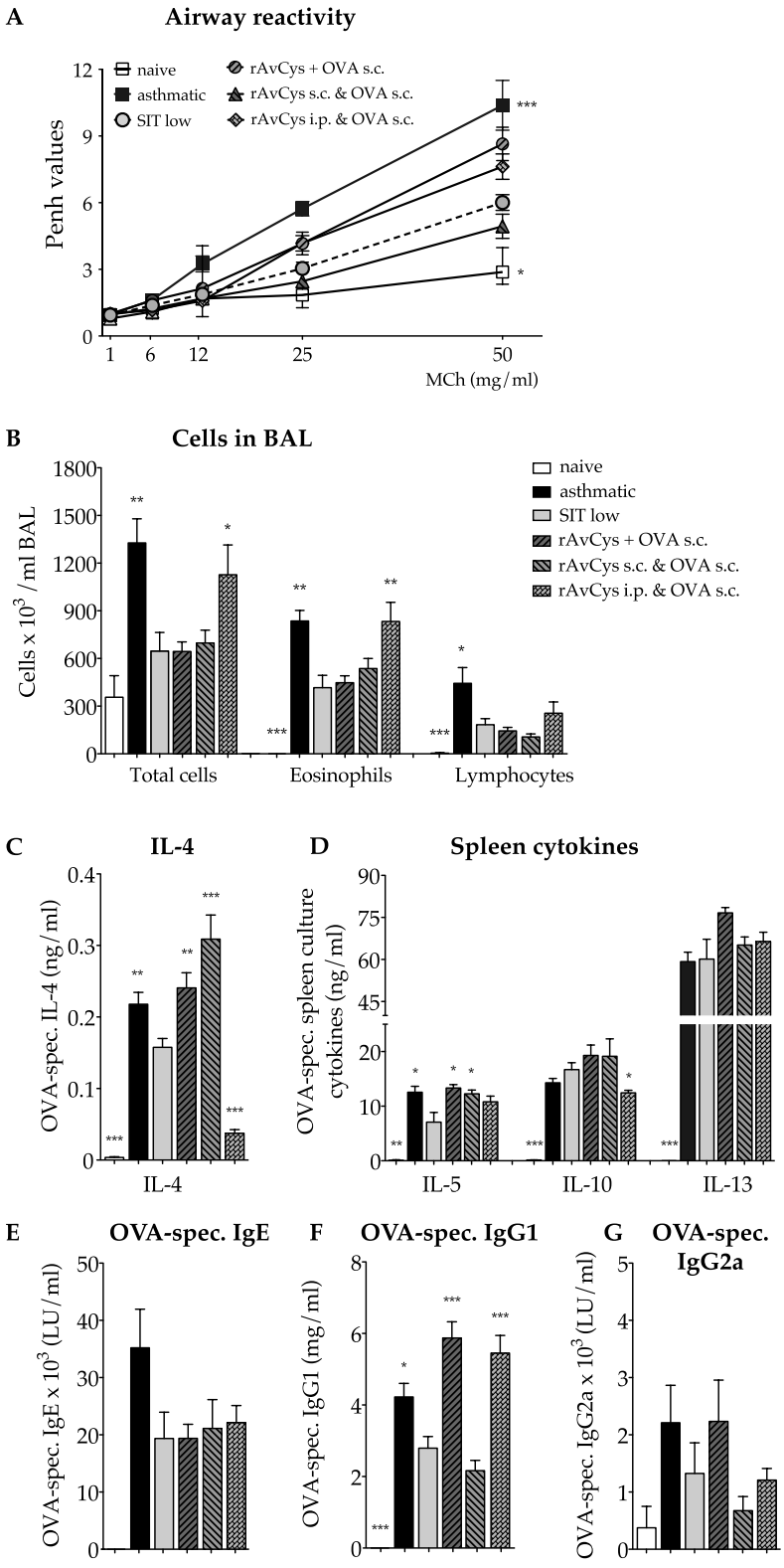

Figure 3.26: Application of rAvCystatin together with SIT low did not boost therapeutic effect of SIT. (A) Airway reactivity. (B) Numbers of total cells, eosinophils and lymphocytes in the BAL fluid. Cytokines: (C) IL-4, (D) IL-5, IL-10 and IL-13 detected in the supernatants from spleen cells restimulated with OVA *in vitro*. (E) OVA-specific IgE, (F) IgG1 and (G) IgG2a levels detected in serum. The mean values ±SEM of one experiment with 4-5 animals per group. Compared with SIT low. *, $p< 0.05$; **, $p< 0.01$; ***, $p<0.001$ are considered statistically significant.

3.4.3 Suppressive capacity of AvCystatin in the OVA-induced immunotherapy model of asthma

As the additive effect of rAvCystatin to specific immunotherapy - SIT low was not observed, it was important to explore whether rAvCystatin by its own has any suppressive capacity in this relatively strong OVA-induced immunotherapy model of allergic asthma, in comparison with SIT treatments. Thus, rAvCystatin was applied alone i.p. as a 'therapy' before challenges on day 28, 30 and 32 as shown in Fig. 3.25.

Measurement of AR by whole-body plethysmography in response to inhaled increasing doses of MCh showed that rAvCystatin applied i.p. before challenges slightly decreased AHR when compared with asthmatic animals ($p=0.12$). However, Penh values from this group remained higher than SIT high or SIT low treatment ($p=0.0005$, $p=0.003$, respectively) (Fig. 3.27A).

Examination of the BAL fluid and cell counts revealed a minor, not significant improvement of the inflammatory cell infiltration into the lungs after rAvCystatin i.p. application (Fig. 3.27B).

It was clear that rAvCystatin had a strong suppressive systemic effect in the OVA-induced model. Levels of OVA-specific Th2-associated cytokines (IL-4, IL-5, IL-10 and IL-13) were significantly decreased after rAvCystatin i.p. treatment, as assessed by examination of supernatants from spleen cells cultured *in vitro* with OVA ($p<0.0001$, $p=0.0005$, $p=0.0007$ and $p=0.01$, respectively) (Fig. 3.27C).

Further, examination of antibodies in serum revealed that rAvCystatin had a capacity to interfere with antibody production. Levels of total IgE and OVA-specific IgE showed a decreased trend ($p=0.08$) (Fig. 3.27B). However, no significant alteration in OVA-specific IgG1 or IgG2a production was observed after rAvCystatin i.p. treatment when compared with asthmatic controls ($p=0.9$, $p=0.6$, respectively) (Fig. 3.27D).

Surprisingly, although SIT high and SIT low reduced all main hallmarks of asthma presented above, allergen-specific immunotherapy did not improve lung pathological changes caused by the allergen. Neither number of eosinophils, lymhocytes nor goblet cell hyperplasia was diminished in the lungs as assessed on H&E and PAS-stained lung sections (Fig. 3.28C, D and J, K). Importantly, rAvCystatin had a downregulatory effect on mucus producing goblet cells. After histological analysis of PAS-stained lung sections, reduced goblet cell hyperplasia was observed in all groups, where rAvCystatin was applied (Fig. 3.28L-N). No clear difference in recruitment of inflammatory cells was observed between lungs of animals from any of the groups treated with rAvCystatin and lungs of asthmatic animals (Fig. 3.28B, E-G). Data show that rAvCystatin applied i.p. exerted partial immunosuppressive effect on the inflammatory responses in the lungs in this model.

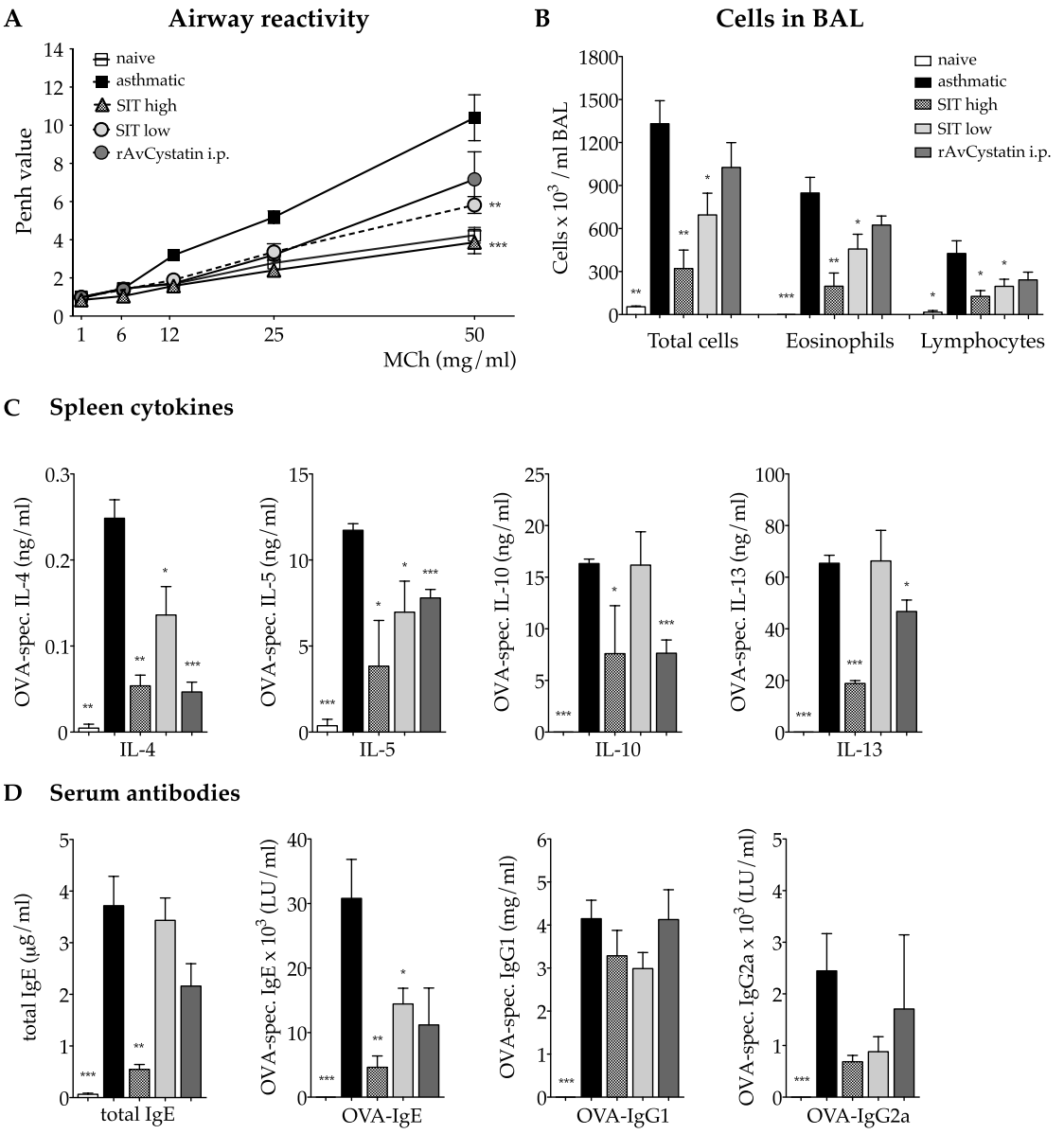

Figure 3.27: Local and systemic responses were altered after rAvCystatin i.p. treatment in the OVA-induced immunotherapy model of allergic asthma. (A) Airway reactivity. (B) Inflammatory cell influx into the BAL fluid. (C) Cytokines detected in spleen supernatants after restimulation with OVA *in vitro*. (D) Antibody levels detected in serum: total IgE, OVA-specific IgE, IgG1 and IgG2a. The mean values ±SEM of one experiment with 5-6 animals per group. Compared with asthmatic controls. *, $p< 0.05$; **, $p< 0.01$; ***, $p<0.001$ are considered statistically significant.

In order to check cytokine response of spleen cells to the stimulation with rAvCystatin *in vitro*, splenocytes from animals of all experimental groups were cultured in the presence of rAvCystatin for 96 hours. Levels of the regulatory cytokine IL-10, Th2-associated IL-13 and Th1-type cytokine IFN-γ were detected in the supernatants.

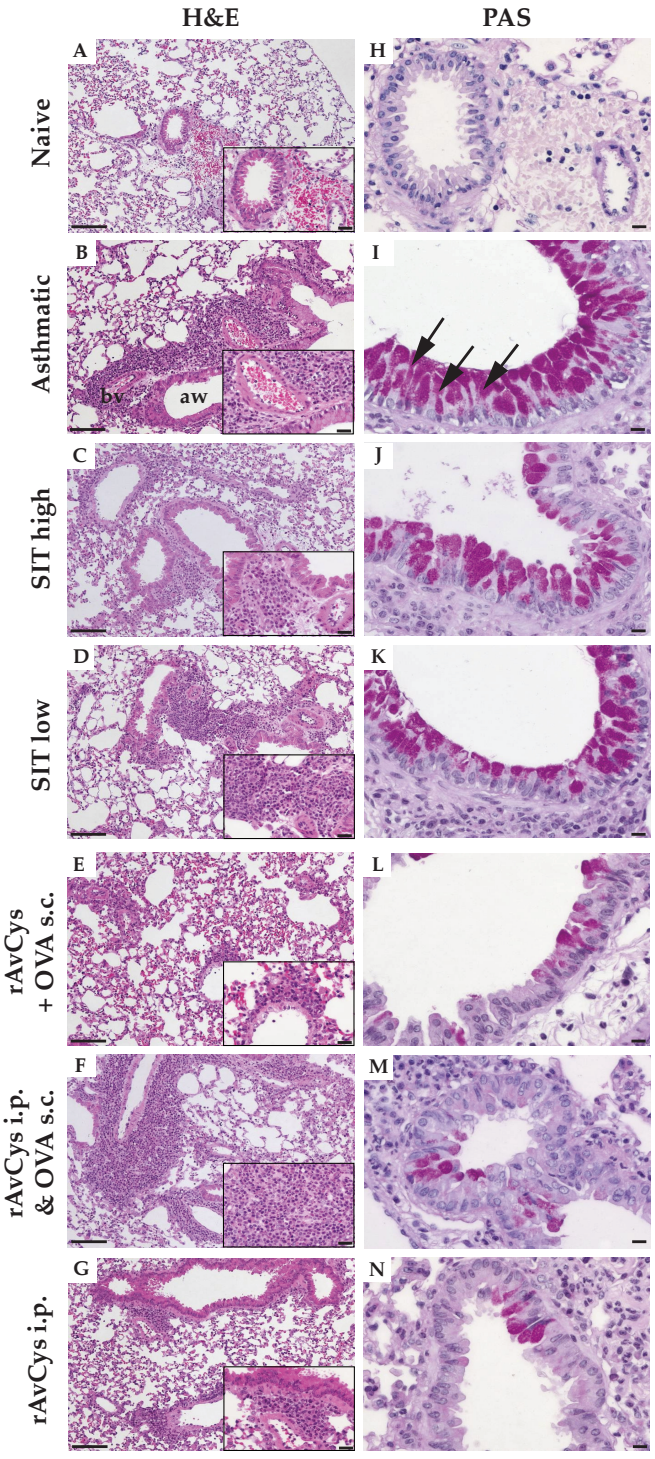

Figure 3.28: Allergen-specific immunotherapy with OVA did not improve the lung pathology. Application of rAvCystatin suppressed goblet cell hyperplasia in the lungs. Representative lung sections stained with (A-G) H&E for eosinophils, in blue: stained nuclei of cells. (H-N) Staining with PAS for analysis of goblet cells, black arrows indicate goblet cells producing mucus. Naive: naive control, asthmatic: OVA sensitized and challenged control; SIT high: OVA high dose; SIT low: OVA low dose; rAvCys + OVA s.c.: rAvCystatin mixed with OVA, applied s.c.; rAvCyst i.p. & OVA s.c.: rAvCystatin treated i.p. & OVA s.c.; rAvCyst i.p.: treated with rAvCystatin i.p. before OVA-challenge. aw: airways; bv: blood vessel. Original magnification: H&E x100 (inset x400); PAS x400; left side scale bars = 100 μm, right side scale bars = 20 μm.

Table 3.3: Cystatin-specific cytokines measured in the supernatants from splenocytes cultured with rAvCystatin *in vitro*.

	Spleen cytokines, mean ±SD (ng/ml)		
Group	IL-10	IL-13	IFN-γ
Naive	0.13 ±0.04	0.01 ±0.002	0.24 ±0.05
Asthmatic	2.36 ±0.65	4.30 ±0.38	0.10 ±0.04
SIT high	0.29 ±0.20	3.17 ±1.17	1.21 ±0.16
SIT low	1.37 ±0.48	2.61 ±1.56	0.33 ±0.31
rAvCys + OVA s.c.	3.27 ±0.26	1.38 ±1.20	1.37 ±0.26
rAvCys i.p. & OVA s.c.	0.40 ±0.27	2.76 ±1.21	nd[a]
rAvCys i.p.	0.72 ±0.03	nd	nd

[a]nd - not detected

High levels of IL-10 were detected in rAvCystatin-restimulated supernatants from asthmatic animals as well as from rAvCystatin + OVA s.c.-group. Cystatin-triggered IL-13 secretion was observed in animals from all groups except from naive and rAvCystatin i.p. alone-treated mice. Increased production of IFN-γ after rAvCystatin treatment was detected in supernatants from rAvCystatin + OVA s.c.-treated group. No IFN-γ was detected in supernatants from spleen cultures of rAvCystatin i.p. & OVA s.c.-group as well as of rAvCystatin i.p. alone-treated animals. It seems that rAvCystatin i.p. application did not lead to any other rAvCystatin-specific cytokine production than the regulatory cytokine IL-10 (Table 3.3).

3.5 Translational model: AvCystatin modulates human allergic responses *in vitro*

In order to assess the clinical potential of rAvCystatin, the aim was to translate the findings from the murine asthma model induced by the clinically relevant allergen - grass pollen into the human system.

To this end the effect of rAvCystatin was tested on peripheral blood mononuclear cells (PBMCs) isolated from clinically characterized, timothy grass (*P. pratense*) pollen allergic patients in comparison to healthy controls, in the context of timothy grass pollen extract (GPE) specific restimulation.

Using human PBMCs stimulated *in vitro* with GPE in the presence of recombinant AvCystatin should reveal its potential as a treatment regimen in allergic humans. In frame of this work rAvCystatin was used for the first time in a clinical setting as a 'proof-of-concept' experiment.

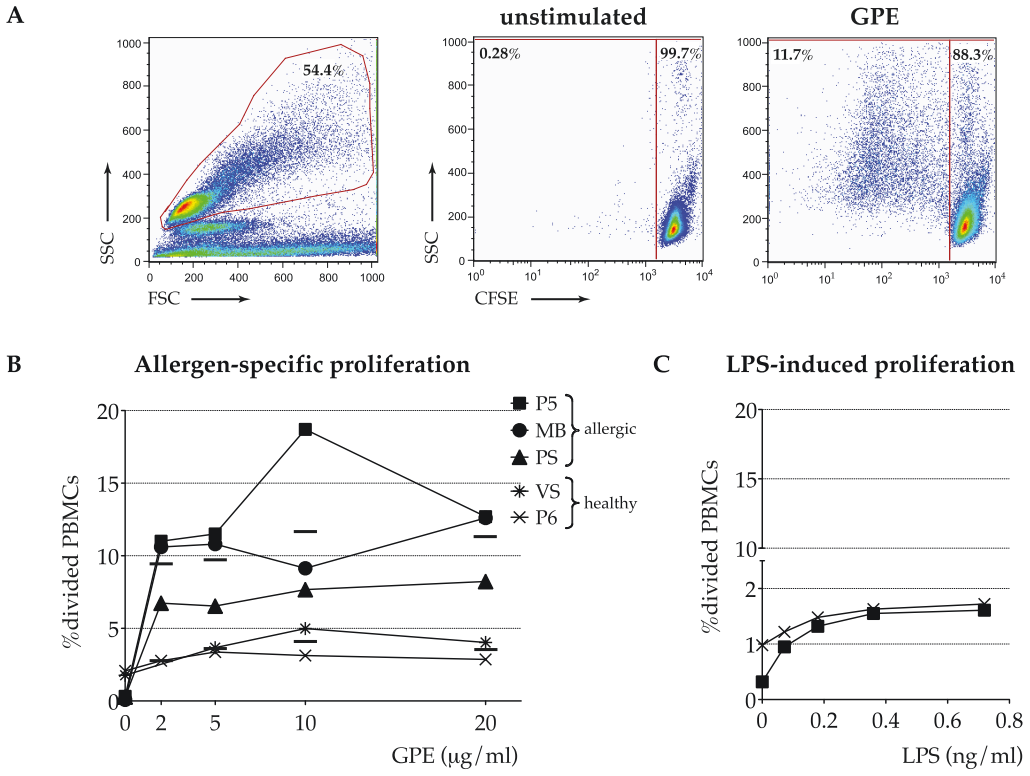

Figure 3.29: Kinetics of lymphocyte proliferation in response to GPE and LPS from allergic and healthy subjects. (A) Gating of CFSE-labeled PBMCs unstimulated and stimulated with GPE. (B) GPE-induced proliferation with increasing doses of GPE at day 7. The mean values ±SEM from allergic subjects and healthy controls are indicated for each GPE concentration. (C) Background influence of LPS on unspecific lymphocyte proliferation. (B, C) % $CFSE^{low}$ divided PBMCs are graphed.

3.5.1 Establishment of an *in vitro* system with human peripheral blood mononuclear cells

As a first approach an *in vitro* system outside of the pollen season was established in order to determine the optimal timing and dosage of allergen and rAvCystatin restimulation.

Grass pollen-induced proliferation of lymphocytes

In order to optimize the culture conditions a primary kinetic experiment with various amounts of GPE was performed. PBMCs were obtained from subjects outside of the pollen season. The most potent proliferation of human PBMCs was observed at day 7 with 5 μg/ml of GPE as assessed by differences in frequencies of CFSE^{low} lymphocytes between three grass pollen allergic subjects and two healthy controls (Fig. 3.29A, B). Such experimental conditions were applied in further approaches.

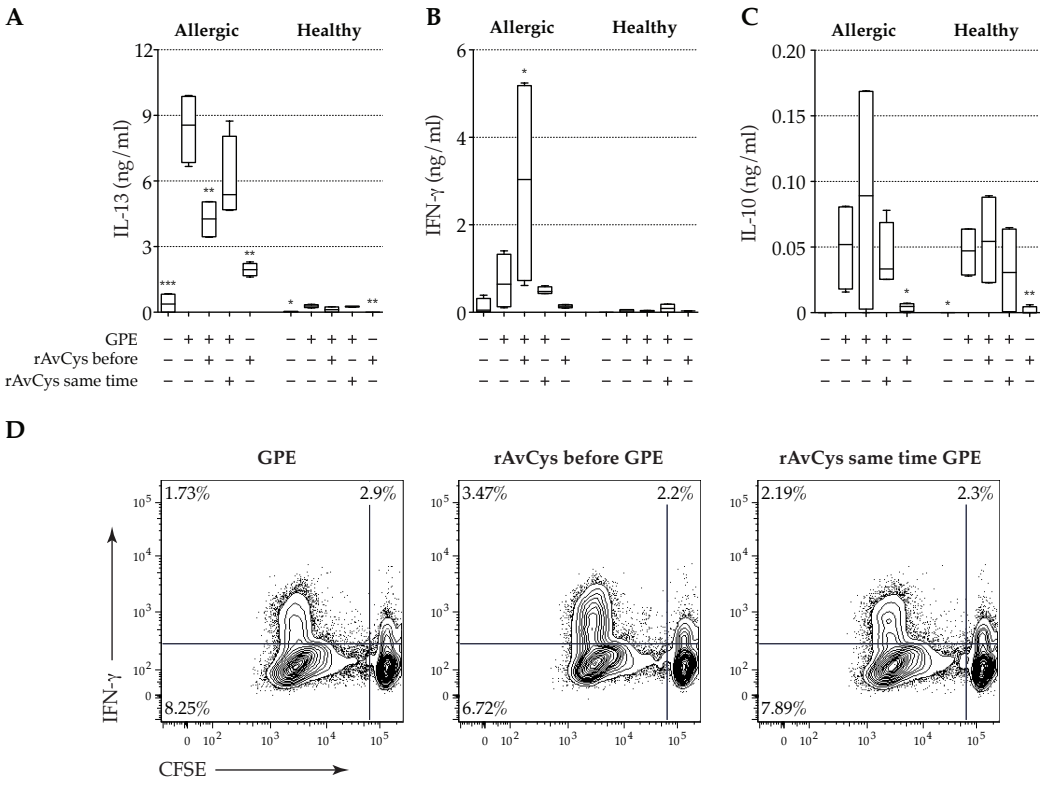

Figure 3.30: Cystatin applied prior to stimulation with GPE reduced production of allergen-specific IL-13 and increased levels of IFN-γ. Levels of (A) IL-13, (B) IFN-γ and (C) IL-10 in supernatants from allergic subjects and healthy controls. The mean values ±SEM from the group. Compared to GPE stimulation. (D) FACS plots with frequency of CD4$^+$IFN-γ^+ proliferating T cells of allergic patients. The mean values from the group as % of CD4$^+$ live T cells. Allergic: N=3; healthy controls: N=2. *, p$<$ 0.05; **, p$<$ 0.01; ***, p$<$0.001 are considered statistically significant.

To analyze an eventual influence of Lipopolysaccharide (LPS) on lymphocyte proliferation, PBMCs were stimulated with corresponding doses of LPS as detected in GPE. LPS induced proliferation of less than 2% PBMCs and there were no differences in response to LPS between lymphocytes from allergic and healthy subjects (1.25 ±0.24 and 1.41 ±0.14, p=0.38, respectively) (Fig. 3.29C). This indicates LPS in GPE had no significant influence on the proliferative responses of human PBMCs.

Time- and dose-dependent effect of AvCystatin

In a preliminary study, culture periods, conditions and doses with rAvCystatin were verified in order to evaluate the most prominent immunosuppressive effect of the protein on Th2-type responses of allergic cells. The application of a lower dose 2.5 μg/ml (0.15 μM) of rAvCystatin (rAvCys low) two hours prior to GPE stimulation (rAvCys low+GPE) resulted in a significant suppression of IL-13 levels and increased IFN-γ production of PBMCs from allergic subjects (p=0.004, p=0.01, respectively) (Fig. 3.30A, B; Table 3.4). Although levels of IL-10 were increased, the differences were not statistically significant when compared with GPE stimulation alone (p=0.49) (Fig. 3.30C; Table 3.4). PBMCs from healthy controls did not show increased IL-13 or IFNγ production after GPE stimulation (Fig. 3.30A, B). However, higher levels of IL-10 from healthy cells stimulated either with GPE alone or rAvCystatin and GPE were observed (Fig. 3.30C).

Table 3.4: Timing and doses of incubation with rAvCystatin *in vitro*: cytokine levels, N=2-3 allergic subjects.

	Cytokines, mean ±SEM (ng/ml)		
Stimulation	IL-13	IL-10	IFN-γ
unstimulated	0.39 ±0.23	nd[a]	0.13 ±0.09
GPE	8.42 ±0.82	0.05 ±0.02	0.69 ±0.32
rAvCys low+GPE	4.26 ±0.45	0.09 ±0.05	2.98 ±1.24
GPE+rAvCys low	6.04 ±0.95	0.04 ±0.01	0.49 ±0.04
rAvCys high+GPE	5.82 ±0.25	0.06 ±0.01	0.87 ±0.14
GPE+rAvCys high	7.05 ±0.38	0.05 ±0.01	0.19 ±0.07

[a]nd - not detected

Furthermore, the frequency of allergen-specific $CD4^+IFN\text{-}\gamma^+$ T cells was increased after stimulation with rAvCystatin prior to GPE (Fig. 3.30D). Stimulation with rAvCystatin alone did not induce proliferation nor cytokine production (data not shown).

In contrary to stimulation with low doses of rAvCystatin before GPE, application at the same time as GPE (GPE+rAvCys low) or incubation with high dose of rAvCystatin (rAvCys high) neither before (rAvCys high+GPE) nor at the same time (GPE+rAvCys high) as GPE showed promising suppressive effects on allergic responses (Table 3.4). Data indicate that rAvCystatin exerted immunomodulatory effect at the specific timing and dose.

3.5.2 Allergen-induced immune responses in the grass pollen season

The effect of rAvCystatin *in vitro* on human T cell immune responses from allergic subjects was further evaluated during the pollen season. To this end, human PBMCs isolated from peripheral blood of clinically characterized patients allergic to timothy grass pollen were examined in the *in vitro* assay as established before.

Clinical characteristics of allergic study subjects

A group of 21 timothy grass pollen allergic subjects was examined in the grass pollen season. Clinical characteristics of the study subjects are indicated in Table 3.5.

All recruited patients showed allergic symptoms (asthma and/or allergic rhinitis). In the serum of all subjects *P. pratense*-specific IgEs were detected. The majority of patients had elevated allergen-specific IgE levels ($\geqslant$ 3.53 kUA/I), which correspond to the defined by ImmunoCAP assay (Phadia AB, Uppsala, Sweden) class of three and higher (reviewed in Cox et al. 2008). Only five subjects had lower allergen-specific IgE levels (range 0.66-1.76 kUA/I) and were ranked as class one or two. On entering the study, subjects were free of oral steroids or immunotherapy for at least 3 months. All of the subjects were otherwise in a good health condition. From the clinical files it was known that most of recruited allergic subjects suffered from other than timothy grass pollen allergies. The most common was allergy to animal dander, HDMs and spring flowers. Only three subjects did not show allergy to anything else than timothy grass.

In order to understand the effect of rAvCystatin on the recall response of allergen-specific T cells, PBMCs from grass pollen allergic subjects were stimulated with GPE and allergen-specific responses were assessed. The aim was to monitor allergen-specific proliferation and to quantify cytokines involved in the Th2 immunity (such as IL-4, IL-5, IL-13) as well as cytokines encompassed in the regulation of the allergic responses, such as IL-10 and IFN-γ. Levels of these cytokines were measured in the culture supernatants after 3 days of GPE-stimulation of PBMCs. Additionally, proliferative and cytokine responses of $CD4^+$ T cells to GPE were measured at day 7 by monitoring of CFSE loss and intracellular cytokine staining in these cells.

Table 3.5: Clinical characteristics of study subjects (N=21)

Patient ID	Age (y)	Gender	Spec. IgE (kUA/I)[a]	Spec. IgE (class)	Other allergies	Smoking	Chronic diseases
01	30	M	17.9	4	None	Y	None
02	29	F	51	5	None	Y	None
03	49	M	30.3	4	None	N	None
04	22	F	0.84	2	Animal dander, HDM[b]	N	None
05	33	M	15.1	3	Animal dander, spring flowers	N	None
06	30	F	31.5	4	Animal dander, celery, peanut, spring flowers, tomatoes, walnut	N	AD[c]
07	25	F	0.98	2	HDM	Y	None
08	33	M	>100	6	Animal dander, HDM, spring flowers	Y	AD
09	20	M	21.2	4	Alder, birch, cat dander, fungi, HDM, herbs, mugwort, tree nuts, rye	N	None
10	25	F	>100	6	Birch, tree nuts	N	None
11	54	M	>100	6	HDM	Y	AD, high blood pressure
12	21	M	43.3	4	Birch, cat dander, HDM	N	None
13	27	F	0.66	1	Honey	N	Urticaria
14	21	F	0.88	2	Horse dander, HDM	N	None
15	33	F	7.33	3	Apple, birch, dog dander, hazelnut, mugwort, pepper, tomato	N	AD
16	42	F	1.76	2	Cat dander, hazelnut, latex	N	None
17	27	F	59.7	5	Animal dander, birch, HDM	Y	AD
18	28	F	5.08	3	Birch, HDM, mugwort, tree nuts	N	None
19	26	F	25.3	4	Spring flowers	N	None
20	22	F	18.1	4	Ambrosia, animal dander, HDM, herbs, molds, rye	N	None
21	41	M	3.53	3	Spring flowers	N	None

[a] kUA/I - kilounits of IgE antibody per liter
[b] HDM - house dust mite
[c] AD - atopic dermatitis

Cytokine and proliferative responses

As assessed by cytokine ELISA, levels of IL-4 and IL-5 were very low in culture supernatants of GPE-stimulated PBMCs, thus the focus was on investigation of IL-13, as well as on IL-10, which could be detected after 3 days. IL-4 and IFN-γ were identified intracellularly after 7 days of cell culture and 5-hour-restimulation with phorbol-myristate-acetate (PMA) and Ionomycin.

In order to confirm that PBMCs of recruited patients were responding to grass pollen extract *in vitro*, analysis of IL-13, IL-10 and proliferation of unstimulated versus GPE-stimulated cells was performed. Stimulation of PBMCs with GPE led to the production of significant amounts of IL-13 and IL-10 in supernatants (p=0.0007 and p<0.0001, respectively) (Fig. 3.31A, B). Allergen-stimulation for 7 days induced proliferation and significantly increased frequencies of proliferating ($CFSE^{low}$) $CD4^+$ $IL\text{-}4^+$ T cells over cells that have not been stimulated with the allergen (p=0.01) (Fig. 3.31C). Moreover, there was a significant positive correlation between GPE-induced proliferating $CD4^+IL\text{-}4^+$ T cells and *P. pratense*-specific IgE levels detected in plasma of patients at the time of enrolling in the study (r=0.74, p=0.02, respectively) (Fig. 3.31D). Additionally, a moderate relationship, however not significant, between $CD4^+$ T cell proliferation and IL-13 production in culture supernatants after GPE-stimulation was observed (r=0.54, p=0.06, respectively) (data not shown).

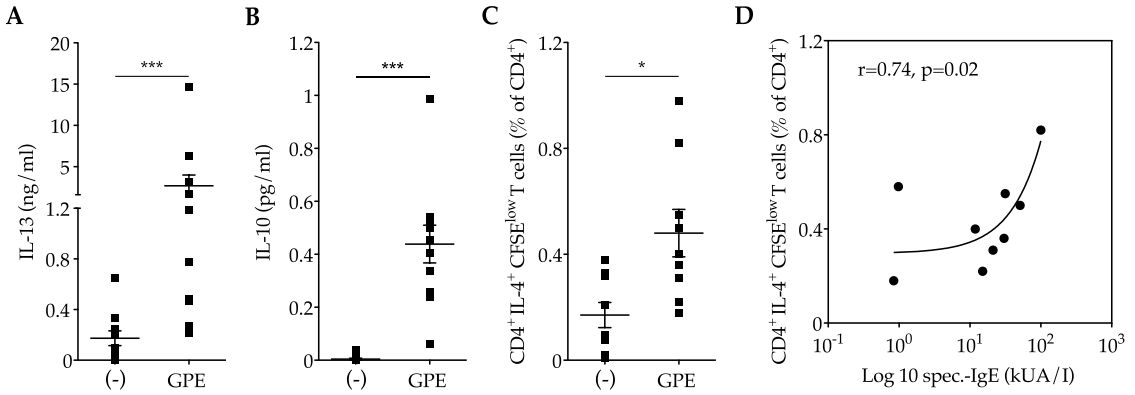

Figure 3.31: Grass pollen-stimulation of human PBMCs from allergic patients induced Th2-type responses. Levels of (A) IL-13 and (B) IL-10 in supernatants. (C) Frequency of $CD4^+IL\text{-}4^+$ proliferating T cells in response to GPE. Patients' responses and mean ±SEM of the group are presented. Compared to values of unstimulated (-) cells. (D) Correlation of $CD4^+IL\text{-}4^+$ proliferating T cells and *P. pratense*-specific serum IgE determined using the Spearman's rank correlation test. The line fitting results was determined by linear regression analysis. Allergen-specific IgE levels are presented on logarithmic scale. (-): unstimulated, GPE: allergen-stimulated. Allergic subjects: N=9-11; r - correlation coefficient; *, p< 0.05; ***, p< 0.001 are considered statistically significant.

Cells positively responded to GPE with increased Th2-type cytokine production and allergen-induced proliferation. Moreover, subjects with higher levels of *P. pratense*-specific IgEs, responded stronger to the GPE-stimulation as shown by production of high levels of IL-4 by allergen-induced proliferating $CD4^+$ T cells. Therefore, the *in vitro* responses of PBMCs from clinically characterized grass pollen allergic patients recruited in the season were positively verified.

3.5.3 AvCystatin modifies human allergen-specific immune responses

To test the effect of rAvCystatin on allergen-specific responses of PBMCs from grass pollen allergic subjects, levels of IL-10, IL-13 and IFN-γ in the supernatants at day 3 of cell culture were quantified. Additionally, allergen-specific proliferation and intracellular cytokine staining was analyzed at day 7 of GPE-stimulation of PBMCs, in the presence or absence with rAvCystatin.

Cytokine secretion

In the *in vitro* system with allergic PBMCs established within this study no significant change in levels of IL-13 between GPE-stimulated PBMCs in the presence or absence of rAvCystatin was observed (1.56±0.72 vs. 1.88±1, p=0.18) (Fig. 3.32A).

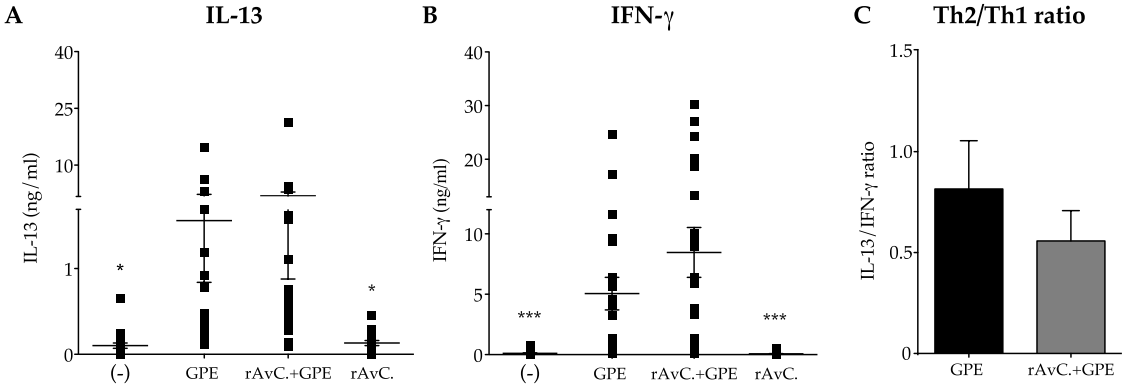

Figure 3.32: Cytokine responses of human PBMCs from allergic patients to rAvCystatin *in vitro*. Levels of (A) IL-13 and (B) IFN-γ in supernatants. (C) Ratio of IL-13/IFN-γ. Patients' responses and the mean values ±SEM of the group are presented. Compared to GPE-stimulation. (-): unstimulated, GPE: allergen-stimulated, rAvC.+GPE: rAvCystatin prior to GPE-stimulation, rAvC.: rAvCystatin-stimulated cells. Total number of allergic subjects: N=21. *, $p< 0.05$; ***, $p<0.001$ are considered statistically significant.

In order to verify whether severity of allergy may influence the effect of rAvCystatin on IL-13 production *in vitro*, subjects were divided into two subgroups according to their *P. pratense*-specific IgE levels: moderate *Pp*-IgE (0-17 kUA/I) and severe *Pp*-IgE (>17.5 kUA/I) (Fig. 3.33A, B). The number of subjects (N) in each subgroup was 9 and 12, respectively. In neither subgroup the presence of rAvCystatin resulted in a statistically significant change of IL-13 production (Δ mean $\pm$SEM GPE vs. rAvCys+GPE, moderate: 0.25$\pm$0.06 vs. 0.32$\pm$0.1, p=0.62; severe: 2.35$\pm$1.2 vs. 2.87$\pm$1.7, p=0.81) (Fig. 3.33A, B; Table 3.6).

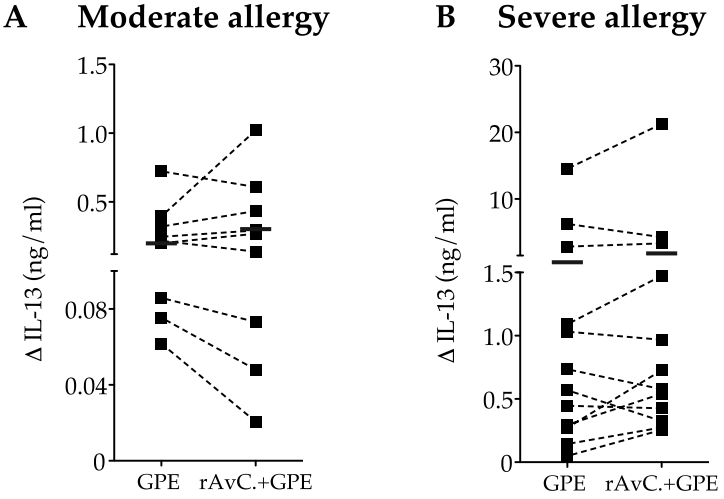

Figure 3.33: Levels of Δ IL-13 of culture PBMCs from patients from the moderate (A) and from the severe (B) allergy group. Patients' responses and the mean values $\pm$SEM of the group are presented. GPE: allergen-stimulated, rAvC.+GPE: rAvCystatin prior to GPE-stimulation. Number of patients in the moderate allergy group: N=9 and in the severe group: N=12.

Levels of IFN-γ showed a trend towards increased production upon stimulation with GPE in the presence of rAvCystatin when compared with GPE-stimulation alone (5.14$\pm$1.4 vs. 8.8$\pm$2.1, p=0.16, respectively) (Fig. 3.32B). This indicates a possible shift in the direction of Th1-driven immunity induced by rAvCystatin as observed with a smaller group of patients out of the pollen season (Subsection 3.5.1).

In the allergic state in humans imbalance in production of Th2 to Th1 cytokines rather than particular amounts might be responsible for the development of allergy (Romagnani, 2004) Analysis of cell culture supernatants revealed that in relation to Th2 cytokine production this trend resulted in a slight conversion from a Th2-biased response to a Th1-biased response in the presence of rAvCystatin.

A Th2/Th1 cytokine ratio was calculated based on IL-13 to IFN-γ ratio in GPE-stimulated PBMCs culture supernatants with or without rAvCystatin. The Th2/Th1 ratio was decreased after treatment with rAvCystatin when compared with GPE-

stimulated PBMCs, however not significantly (p=0.36) (Fig. 3.32C). This can be explained by the fact that no significant change in IL-13 production was observed after *in vitro* application of rAvCystatin prior to GPE-stimulation (Fig. 3.32A), but there was a considerable increase in IFN-γ in the presence of rAvCystatin (Fig. 3.32B). The Th2/Th1 ratio was substantially decreased after application of rAvCystatin when compared with GPE-stimulation alone (Fig. 3.32C). Further analysis of 3-day culture supernatants showed no significant change in levels of IL-10 of GPE-stimulated PBMCs in the presence of rAvCystatin when compared with supernatants of GPE-stimulated PBMCs (data not shown).

Table 3.6: Cytokine response to allergen and rAvCystatin; subgroup mean ±SEM. Moderate and severe subgroup of allergy, in total examined N=21 allergic subjects.

		Allergy subgroups	
Cytokines (pg/ml)	**Stimulation**	**Moderate** (0-17 kUA/I)[a], N=9	**Severe** (>17.5 kUA/I), N=12
IL-13	Unstimulated	56 ±9	120 ±42
	GPE	314 ±71	2064 ±988
	rAvCys+GPE	378 ±111	2523 ±1379
	rAvCys	82 ±34	291 ±138
IFN-γ	Unstimulated	192 ±89	19 ±10
	GPE	3588 ±1101	6304 ±2314
	rAvCys+GPE	5725 ±1480	11110 ±3493
	rAvCys	120 ±61	20 ±7
IL-10	Unstimulated	9 ±5	4 ±3
	GPE	562 ±112	434 ±73
	rAvCys+GPE	592 ±104	349 ±60
	rAvCys	14 ±13	2 ±1

[a]kUA/I - kilounits of IgE antibody per liter

Intracellular allergen-specific cytokine secretion

Under conditions of optimal GPE concentration a significant increase of proliferation ($CFSE^{low}CD4^+$ T cells) was detected at day 7 of cell culture when compared with unstimulated PBMCs (p=0.02) (Fig. 3.34A). However, stimulation with rAvCystatin and allergen did not alter the percentages of grass pollen-reactive $CD4^+$ T cells that proliferated (p=0.8) (Fig. 3.34A).

In order to evaluate the effects of rAvCystatin on the cytokine patterns of individual allergen-responding T cells, an intracellular staining for the cytokines IL-4, IL-10 and IFN-γ on the single cell level at the day 7 of culture was performed. Incubation with GPE led to a significant increase of frequencies of $CD4^+$IL-4^+ T cells

when compared with unstimulated cells (p=0.03) (Fig. 3.34B). Moreover, treatment with rAvCystatin significantly reduced allergen-specific $CD4^+IL\text{-}4^+$ T cells when compared with stimulation with the allergen alone (p=0.04) (Fig. 3.34B).

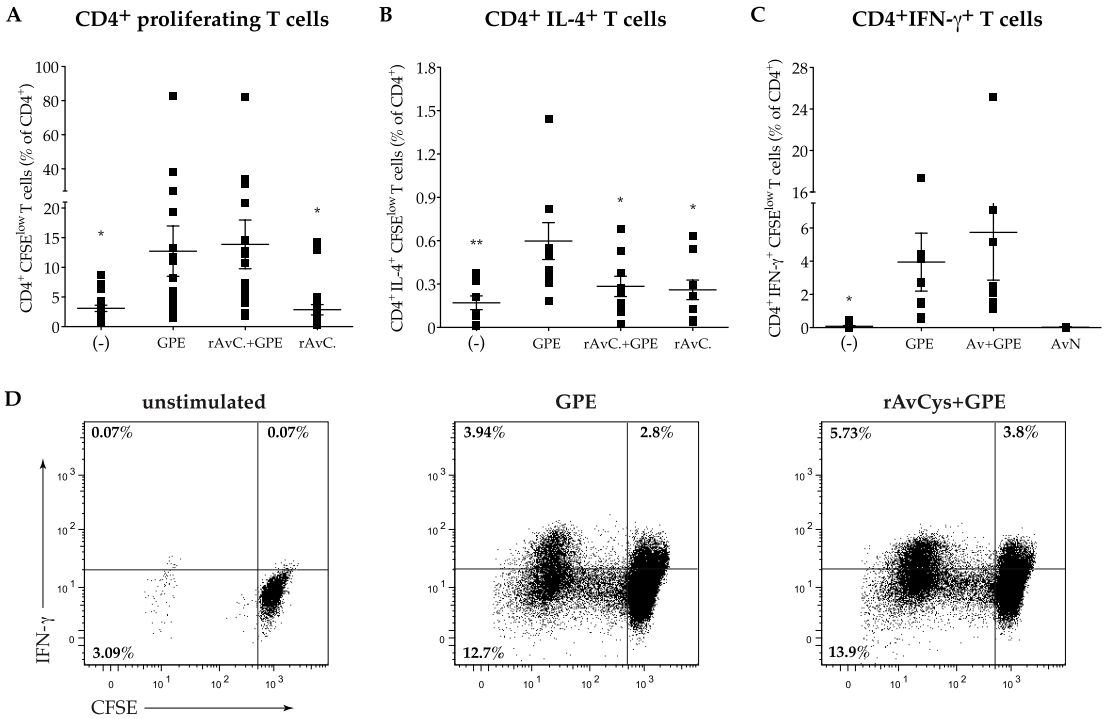

Figure 3.34: Responses of human PBMCs from allergic patients to rAvCystatin stimulation. Frequencies of (A) $CD4^+$ T cells (B) $CD4^+IL\text{-}4^+$ T cells and (C) $CD4^+IFN\text{-}\gamma^+$ T cells proliferating in response to GPE. Patients' responses and the mean values ±SEM of the group are presented. (D) Representative FACS plots with frequency of $CD4^+IFN\text{-}\gamma^+$ T cells of allergic patients. The mean values from the group are presented as % of $CD4^+$ live T cells. Compared to GPE-stimulation. (-): unstimulated, GPE: allergen-stimulated, rAvC.+GPE: rAvCystatin prior to GPE-stimulation, rAvC.: rAvCystatin-stimulated cells. Total number of examined allergic subjects: N=9. *, $p< 0.05$; **, $p< 0.01$ are considered statistically significant.

Increased, however not significantly, percentage of proliferating $CD4^+IFN\text{-}\gamma^+$ T cells was detected after treatment with rAvCystatin (p=0.5) (Fig. 3.34C, D). This is in line with the increased levels of IFN-γ in the culture supernatants after 3-day stimulation with GPE in the presence of rAvCystatin (Fig. 3.32B). In case of allergen-specific $CD4^+IL\text{-}10^+$ T cells, there was no substantial change in the percentage of those cells after treatment with the immunomodulator (data not shown).

Data show that rAvCystatin in the grass pollen season, although not changed levels of allergen-specific IL-13, is able to downregulate IL-4 cytokine responses of allergen-specific $CD4^+$ T cells, which is in line with induced levels of IFN-γ that possibly generate the inhibitory effect on allergen-reactive $CD4^+IL\text{-}4^+$ T cells.

4. Discussion

4.1 Mast cells and Th2 response

Both parasites and allergens induce Th2-driven immunopathologies. The key players in Th2-type immunity are effector $CD4^+$ Th2 cells producing signature Th2 cytokines (IL-4, IL-5, IL-10 and IL-13). Th2-associated cytokines are essential for the differentiation, survival and activity of MCs, basophils, eosinophils, neutrophils and mucus producing cells as well as for the tissue homing of Th2 cells and for production of the antibody isotypes like IgG4 and IgE (Maizels and Yazdanbakhsh, 2003; Finkelman et al., 2004; Anthony et al., 2007; Saenz et al., 2008). Additionally, novel tissue-derived cytokines with proinflammatory functions, such as IL-25, IL-33 and TSLP were described to initiate and regulate Th2 immune response in helminth infections as well as in allergy (Bilsborough et al., 2006; Wang et al., 2007; Goswami et al., 2009; Massacand et al., 2009; Liew et al., 2010; Iwakura et al., 2011; Medzhitov et al., 2011). MCs are involved in type 2 immune responses and typically have been regarded as effector cells in later stages of helminth infections and allergy, while their task in Th2 priming has been relatively neglected. The present study reveals a role of MCs in the early events of Th2 immunity induced by parasites as well as the function of tissue-derived cytokines.

Two independent mouse models of MC-deficiency showed that mice lacking MCs were unable to develop proper Th2 immunity, failed to expulse worms and to control parasitic egg production. This was observed in the present study for the infection with the duodenal helminth *H. polygyrus* as well as the ceacal dwelling nematode *T. muris*. Bone marrow (BM) 'repair' of the MC-compartment led to restoration of the anti-parasitic responses and Th2 priming. It has been shown that transfer of whole BM but not bone marrow-derived-cultured MCs (BMMCs) restored mucosal MCs in the intraepithelial layer of the intestinal tract. *In vitro* generated MCs tend to appear as too immature and inactive to properly restore their tissue distribution and function in the small intestine and give a raise to different types of MCs, depending on the site of development (Abe and Nawa, 1987). Nakano et al. (1985) showed that i.p. transfer of BMMCs successfully restored MCs populations in the peritoneal cavity, spleen, skin and stomach of Kit^W/Kit^{W-v} mice. Interestingly, no restoration of MCs in the small intestine and some other organs after i.p. or i.v.

BMMCs injections has been reported (Nakano et al., 1985; Tanzola et al., 2003). Additionally, many studies circumvent problems associated with BMMCs reconstitution in the small intestine by transfer with whole BM, and such approach has been accepted when investigating the role of MCs in the small intestine (Ierna et al., 2008; Sakata-Yanagimoto et al., 2011). However, in general it is difficult to predict whether engrafted cells can restore all functions of the corresponding populations of MCs existing in wild type mice. The most convincing and widely accepted argument about a particular role of MCs is when transferred and engrafted BM, BMMCs or *in vivo*-derived MCs convert the phenotype of MC-deficient mice similarly to that observed in WT animals (Kawakami, 2009).

Although transfer experiments with whole BM might not be MC-specific, in the present study it is unlikely that BM transfer, even if bringing type 2 producing cell progenitors (e.g. multipotent progenitor type 2), restored Th2 immunity independently of MCs reconstitution. Saenz et al. (2010) showed that the accumulation of multipotent progenitor (MPP) type 2 cell population in the gut-associated lymphoid tissue was absolutely dependent on IL-25 expression. With such a signal MMP(type 2) cells were able to promote Th2-cytokine responses. In the present study no IL-25 levels were observed in Kit^{W}/Kit^{W-v} mice upon helminth infection, thus this cell progenitors were unlikely to be responsible for the restoration of the phenotype in MC-deficient mice. Therefore, it is probable that the absence/inactivity of cell progenitors is caused by a lack of IL-25 expression following infection in MC-deficient mice.

Furthermore, it was evident that MCs were involved in regulating the tissue-derived cytokines IL-25, IL-33 and TSLP during the first days of helminth infection with *H. polygyrus* and *T. muris*. Fort et al. (2001) proved the relevance of IL-25 in induction of Th2 immune responses. Exogenous administration of IL-25 led to expression of IL-4, IL-5 and IL-13, eosinophilia and increased IgE levels, thus amplifying allergic type inflammatory responses by its actions on accessory cells that were $MHCII^{high}$ $CD11c^{dull}$ and $lineage^{-}$. Moreover, Zhao et al. (2010) showed that mice treated with IL-25 exhibited strong induction of Th2-derived cytokines and changes in the function of the intestinal tract including enhanced smooth muscle hyper contractility and enhanced mucosal permeability. In frame of the present work, reconstitution of MC-deficient animals with rIL-25 reversed the Th2 impairment and led to worm expulsion and decreased egg burden, pointing to the importance of MCs and tissue-derived cytokines in early Th2 immune responses in helminth infections. It became clear that MCs residing in the intestinal mucosa are required for the induction of IL-25 in the tissues leading to mucosal homeostasis. As it was not possible to directly prove that MCs are the source of IL-25 during infection with *H. polygyrus*, it cannot be excluded that MCs contribute to the production of tissue-derived

cytokines via other mechanisms. MCs are able to produce various pro-inflammatory molecules, which are released from pre-stored granules. It is very probable that a certain pre-formed mediator released from MCs gives an adjuvant boost to epithelial cells and/or other cells for expression of IL-25/IL-33/TSLP cytokines. It would be very interesting to investigate the following questions: What exact mediator is responsible for this effect? Which cells are responding to MC-specific signals by early production of tissue-derived cytokines? Are there any other cells that together with MCs orchestrate Th2 immunity during helminth infection but also during allergic reactions?

The data in this study on MC-deficient mice as well as reconstitution experiments provide an evidence for a critical involvement of MCs in priming of Th2 responses. Thus, this work shows that MCs orchestrate protective Th2 immunity upon helminth infection (Hepworth et al., 2012). Moreover, current understanding of the early role of MCs in type 2 responses could be applied in the prevention of the development of other Th2-driven inflammatory conditions such as asthma and allergies.

4.2 Influence of the helminth immunomodulator AvCystatin in the model of allergic asthma

Allergic asthma induced by the clinically relevant allergen

Animal models of asthma present an excellent experimental tool to investigate allergic disorders and to evaluate possible treatments *in vivo*.

In frame of this thesis an improved mouse model of airway hyperreactivity and inflammation induced by the clinically relevant human aeroallergen, timothy grass (*P. pratense*) pollen, was established. Three sensitizations with the recombinant major pollen allergen group V isoform b (rPhl p 5b) followed by challenges with standardized timothy grass pollen extract (GPE) led to all hallmarks of allergic airway hyperreactivity, including inflammatory cell influx into the BAL fluid, local and systemic induction of Th2-associated cytokines and increased levels of allergen-specific antibodies. Isoform b of Phl p 5 (Phl p 5b) was described to be one of the most reactive allergens from the group V of grass allergens, partially due to bearing at least one more IgE antibody binding epitope than an isoform a (Phl p 5a) (Andersson and Lidholm, 2003). The major timothy grass pollen allergen group V is an RNAse involved in the plant's defence against infections. Contribution of plant RNAses in the development of allergies in human is so far unknown in contrast to other allergens exhibiting proteolytic activity that contribute and enhance allergic responses, like the major HDM allergen (Der p 1) or papain allergen (Car p 1), acting

as cysteine proteases (Chapman et al., 2007; Gunawan et al., 2008; Cunningham et al., 2012).

In order, to establish an improved model induced by the grass pollen allergen, the BALB/c mouse strain was chosen. In line with other studies, a very successful induction of allergic asthma was achieved in this mouse strain in our study. Seitzer et al. (2005) showed that BALB/c but not C57BL/6 or SJL/j mice were efficiently responding to timothy grass pollen allergens and were qualified as 'high' responders to *P. pratense* grass pollen. The same group reported earlier that the IgE response of BALB/c mice to *P. pratense* extract and allergens resembles the one observed in allergic human patients. The only difference to humans was that BALB/c mice did not react to Phl p 1 that most of the human patients respond to (96%) followed by a response to Phl p 5 (80%) and Phl p 6 (75%) allergens (Niederberger et al., 1998; Vrtala et al., 1999; Rossi et al., 2000; Seitzer et al., 2003). Additionally, the sensitization with a single allergen like Phl p 5b gives a possibility to produce a fusion protein with any other molecule that would serve as a treatment in the future and would enable such a chimeric construct to target allergen-specific Th2 cells. For these reasons recombinant Phl p 5b, as a clinically relevant allergen, was chosen for sensitization of BALB/c mice in frame of this thesis.

Numerous studies report on sensitization with OVA antigen by intraperitoneal (i.p.) (Hamelmann et al., 2000; Whitehead et al., 2003), subcutaneous (s.c.) (Corry et al., 1996; Faustino et al., 2012), intranasal (i.n.) (Mori et al., 2011) and intratreacheal (i.t.) (Mizutani et al., 2010) delivery. In this regard, i.p. injections were reported to induce higher production of allergen-specific IgEs than i.n. sensitization (Zhang et al., 1997). However, there is not so much information available on different ways of sensitization to grass pollen allergens in mice and the best described route so far is i.p. injection (Darcan et al., 2005; Wallmann et al., 2010). In the present study challenge was achieved by i.n. applications of whole pollen grass extract (GPE). Such delivery mimics the way humans are being challenged in the pollen season when getting in contact with the aeroallergen. Some studies report on use of recombinant Phl p 5 for a successful i.n. challenge (Darcan et al., 2005; Gabler et al., 2006). However, considering the cost of recombinant allergens and the fact that extracts apart from various allergens bear other components like oxidases or bioactive lipid mediators (PALMs), which act as 'natural adjuvants' and contribute to Th2-polarization and development of allergic responses (Plötz et al., 2004; Boldogh et al., 2005; Traidl-Hoffmann et al., 2005), the whole pollen extract was chosen in this study.

Grass pollen extracts from different companies show relatively big heterogeneity regarding the presence of individual allergens (Focke et al., 2008). In frame of this work, all experiments were performed with a standarized batch of GPE

with a defined content of Phl p 5 per amount of grass extract (6.09 ng/PNU as 38.5 μg/mg). Numerous studies describe the usage of adjuvants, i.e. suspensions of aluminum hydroxide (Alum) or magnesium hydroxide that promote and enhance Th2-inflammatory responses (Kumar et al., 2008). Nakae et al. (2007) showed that sensitization with OVA/Alum develops IgE- and MC-independent AHR and airway inflammation in contrast to MC-driven allergic inflammation when OVA was administered without Alum. Potentially, dropping usage of non-natural adjuvants in the future would closer resemble allergic development as it happens in natural sensitization and challenge in humans.

Besides experimental models using OVA or grass pollens to induce allergy in mice, there are a few other, alternative models being developed. Vrtala et al. (2000) reported that four sensitizations with the major pollen allergen group I of *Betula verrucosa* (Bet v 1) over 94 days adsorbed to Complete Freund's Adjuvant, led to strong induction of IL-4, IL-5 and IL-13, as well as production of allergen-specific antibodies. Batanero et al. (2002) sensitized BALB/c mice with native olive allergen Ole e 1 (nOle e 1) and recombinant Ole e 1 (rOle e 1) together with Alum. Olive allergens induced high levels of specific IgE and IgG1 versus low IgG2a. Splenocytes from olive-sensitized mice exhibited a proliferative response to nOle e 1. Allergen-specific IL-4, but no IFN-γ, was detected (Batanero et al., 2002). A novel mouse model using cockroach (rBla g 2) and HDM (rDer f 1) allergens - 'inner city' environment allergens - was developed by Sarpong et al. (2003). After two i.p. sensitizations with allergens/Alum followed by a single challenge via a tracheal catheter, A/J mice exhibited a strong influx of inflammatory cells, especially when the two allergens were applied together. However, the cockroach allergen but not HDM induced airway hyperreactivity in allergic mice (Sarpong et al., 2003). In a recent study Murdock et al. (2012) demonstrated that four i.n. challenges with *A. fumigatus* in C57BL/6 mice caused chronic pulmonary inflammation characterized by eosinophilia, goblet cell metaplasia and Th2-associated cytokine production.

In this study sensitization and challenge with the clinically relevant grass pollen allergen and pollen extract induced high numbers of total inflammatory cells and eosinophils into the lungs when compared with an OVA-induced model. The differences in strength of Th2-immunological parameters between the two models may be caused by a stronger allergenicity of rPhl p 5b as a clinically relevant allergen as well as due to the fact that animals were sensitized with three i.p. rPhl 5b-injections versus two i.p. OVA-sensitizations. In line with that, expanded contribution of Th2-cytokines, especially IL-4 and IL-13 led to increased (more than in the OVA-model) levels of total and rPhl p 5b-specific antibodies.

Indeed, IL-4 and IL-13 stimulate multiple features of asthma like eosinophilia, IgE production, goblet cell metaplasia, AHR, smooth muscle remodeling, and overall Th2

induction and maintenance. IL-4 binds to the type I and II IL-4 receptor (IL-4R). Whereas IL-13 selectively targets IL-4RII (composed of the IL-4Rα and IL-13Rα1 chains), which all together induce IL-4Rα-mediated activation of the transcription factor Stat6 and upregulation of the expression of GATA-3, the master regulator for the Th2-differentiation (Zhu et al., 2001; Paul and Zhu, 2010). Therefore, on the one hand the roles of IL-4 and IL-13 in the development of allergic airway disease overlap. But on the other side, IL-4 but not IL-13 stimulates T cells, B cells and MCs, and directs naive T helper cells into Th2-biased responses (Nelms et al., 1999; Finkelman et al., 2010). Moreover, IL-4 mediates antibody class switch on B cells to IgEs, enhances tissue homing of inflammatory effector cells, and induces expression of endothelial vascular cell adhesion molecule (VCAM)-1, which directs eosinophils to sites of inflammation (Oliphant et al., 2011; Williams et al., 2012). Interestingly, it was shown that IL-13Rα1 regulated baseline IgE, which was independent of changes in IL-4. Additionally, IL-13Rα1 was proposed to be the key regulator of IL-13- and OVA-induced TGF-β production, that in contrary to regulatory TGF-β (Taylor et al., 2006), leads to allergen-driven fibrotic reactions (Munitz et al., 2008).

However, a development of airway hyperreactivity and eosinophilia is not exclusively regulated by IL-4 or IL-13. In our study airway reactivity of asthmatic mice, numbers of eosinophils and levels of IL-5 were highly increased when compared to naive animals and were comparable to each other between rPhl p 5b- and OVA-induced models. IL-5 plays a central role in the induction of eosinophilia and AHR. IL-5-transgenic mice have been reported for high numbers of eosinophils in the peripheral blood and organ eosinophilia (Dent et al., 1990). Studied by two groups, anti-IL-5 mAb treatment of allergic mice suppressed AHR (Hamelmann et al., 1997a; Hogan et al., 1997a). Within the last years, there have been several clinical trials conducted using anti-IL-5 antibodies (mepolizumab and reslizumab) or anti-IL5R (benralizumab) to prove their efficacy and safety in patients mostly with severe asthma (reviewed in Corren, 2012). From the animal data it is known that allergen-sensitized and challenged IL-5-deficient mice, lacking production of IL-5, showed no significant eosinophilic airway inflammation, which was associated with no change in the development of airway responsiveness to methacholine (Hamelmann et al., 2000). Therefore high levels of Th2-cytokines (namely IL-4, IL-5 and IL-13) in our model surely contributed to the strong development of all hallmarks of allergic asthma induced by the clinically relevant aeroallergen.

AvCystatin's effect in the murine model with the clinically relevant allergen

There is an evidence from field studies with helminth-infected individuals on a negative correlation between allergic Th2-based immunopathologies and incidence of helminth infections. Additionally, this is supported by reports on experimental animal models (reviewed in Daniłowicz-Luebert et al., 2011). One of the aims of this thesis, based on previous work (Schnoeller et al., 2008), was to evaluate the bystander effect of a single parasitic cysteine protease inhibitor from the rodent filariae *A. viteae*, AvCystatin (cystatin), in the mouse model of allergic asthma.

Indeed, this work shows that AvCystatin interfered with allergen sensitization and minimized the effect of challenges with grass pollen extract. Airway hyperreactivity (AHR) assessed *in vivo* was significantly reduced after treatment with AvCystatin when compared with airway reactivity of asthmatic animals and, at the same time, was on the level of naive mice.

Downstream the 'allergic cascade' effector cells such as eosinophils, B cells and MCs are being recruited into airways mainly through Th2-cytokine sensing. AvCystatin was able to interfere not only with inflammatory cell recruitment into the lungs but also with local and systemic production of Th2-cytokines (such as IL-4 and IL-5) and IgE levels. Suppression of above possibly contributed to decreased AHR. Other authors showed that depletion of Th2-cytokine-producing $CD4^+$ and $CD8^+$ T cells hindered eosinophil infiltration into the airways and the development of AHR (Gavett et al., 1994), additional lack of $CD8^+$ T cells abrogated local production of IL-5 by the lymph nodes (Hamelmann et al., 1996). Moreover, reconstitution with IL-5 increased number of eosinophils and restored AHR of OVA-sensitized and challenged IL-4-deficient mice (Hamelmann et al., 2000). Thus, possibly reduction of IL-4 and IL-5 after treatment with AvCystatin contributed to a suppressed AHR.

Additionally in the present study, AvCystatin decreased local and systemic production of IL-13. This was in contrast with a previous work on the OVA-induced model where AvCystatin did not alter IL-13 production (Schnoeller et al., 2008). It cannot be excluded that decrease of IL-13 mediated by AvCystatin, could be solely responsible for reduced AHR in the timothy grass pollen-induced model. One explanation for this might be that IL-13 independently of IL-4, IL-5 and eosinophilia induces AHR as it was shown in several studies. Mice lacking IL-13 did not develop allergen-induced AHR, although other Th2 cytokines and eosinophilic pulmonary inflammation were present (Hogan et al., 1997b). Administration of exogenous recombinant IL-13 restored AHR in $Il13^{-/-}$ animals. Moreover when SCID mice were transfered with OVA-specific Th2 cells from OVA TCR-transgenic $Il13^{-/-}$ mice, they failed to induce AHR, although $Il13^{-/-}$ Th2 cells were capable to produce IL-4

and IL-5 (Walter et al., 2001). Interestingly, in the present work levels of allergen-specific IL-13 were around 20 - 50 fold higher over levels of IL-4, detected in supernatants of allergen-restimulated PBLNs and spleen cells of AvCystatin-treated animals but significantly lower than levels detected in asthmatic animals. Rothenberg et al. (2011) also reports on increased IL-4/IL-13 ratios in HDM-induced model of allergy. As anti-IL-13 reagents are under clinical trials for asthma, findings on the capacity of AvCystatin to suppress IL-13 bear promising potential. Whether AvCystatin acts e.g., via blockade of IL-13Rα, might be approached in further studies.

Effect of AvCystatin on mast cells

Furthermore, AvCystatin suppressed local recruitment of MCs and reduced levels of MC-protease therefore interfered with MC-activation. One mechanism might be that AvCystatin may suppress IgE production via decreasing levels of IL-4 and IL-13. This can lead to restrained IgE binding to high-affinity receptor FcϵRI on MCs and to further reduced allergen-triggered MC-degranulation and release of preformed mediators like MC-proteases (i.e. mMCP-1) or histamine. Such mediators are described to contribute to tissue remodeling and influence cellular recruitment (Marshall and Jawdat, 2004). A histamine receptor-1 has been shown to play an important role in the development of AHR, airway inflammation and Th2-cell recruitment to sites of allergic inflammation (Bryce et al., 2006). Other authors showed that treatment with histamine-1-receptor (H1R)-antagonists before or during sensitization with OVA prevented eosinophilic inflammation and development of AHR (Blumchen et al., 2006).

Another mechanism of AvCystatin acting on MCs might be cross-linking of FcϵRI with the low-affinity IgG receptor (FcγRIIb), although it has not been proven yet. However, it is known that AvCystatin is able to form dimers and oligomers when the additional N-terminal cysteine is present. Therefore such scenario would be feasible as then AvCystatin could act on these two receptors simultaneously. This would interfere with signal-transduction cascade of MCs leading to the inhibition of MC-mediator release. Cross-linking of the FcϵRI-IgE complex on the MC-surface with the FcγRIIb-IgG complex was shown to lead the suppression of FcϵRI signaling and MC-activation through FcγRIIb immunoreceptor tyrosine-based inhibition motif (ITIM) phosphorylation by FcϵRI-associated activation (Kraft and Novak, 2006).

Furthermore, Zhu et al. (2005) and Eggel et al. (2011) reported successful coaggregations of FcϵRI and FcγRIIb by produced chimeric fusion proteins of a part of human IgG1 with either a cat allergen (Fel d 1) or a genetically engineered antibody mimetic protein (DARPin). Moreover, Melendez et al. (2007) reported a filarial nematode protein ES-62 to reduce FcϵRI-mediated responses of human and murine

MCs. However, whether MCs play a critical role in the induction of chronic asthma in the clinically relevant timothy grass pollen model is unclear. Related animal models that use sensitization with model allergens, such as OVA, mixed with Alum (Williams and Galli, 2000) do not require MCs whereas development of disease in mice exposed to repetitive i.n. antigen challenges in the absence of Alum was shown to be MC-dependent (Nakae et al., 2007). To further study effects of AvCystatin on the interference with MCs and resulting downmodulation of allergy, it would be interesting to use an adjuvant-free protocol to achieve MC-dependent sensitization in the grass pollen-induced model or/and to employ MC-deficient mice strains or to perform experiments with sensitized/non-sensitized MCs *in vitro*.

Another plausible activity of AvCystatin interacting with MCs could be in the beginning of Th2 development during sensitization phase. As revealed in the first part of this work, MCs orchestrate Th2 immunity in helminth infections by regulation of early cytokines like IL-25, IL-33 or TSLP (Hepworth et al., 2012) and play a key role in an initiation of allergic responses (reviewed in Williams et al., 2012). Although not proven here, in such situation AvCystatin may not only act as a later-stage suppressor of MCs but also may hinder early activation of MCs through such cytokines or may block adjuvant stimuli of MCs on activation of cells producing IL-25, IL-33 and TSLP, that are important initiators of Th2 responses and play a role in the development of allergies (Smithgall et al., 2008; Corrigan et al., 2011).

Additionally, AvCystatin could also interfere with MCs through increased numbers of Treg cells and production of IL-10. Treg cells were described to regulate MCs activity by altering expression and signaling of IgE receptor and by producing IL-10 to silence their function (Kashyap et al., 2008; Kennedy Norton et al., 2008). Moreover, IL-10 diminishes IgE expression and favors antibody subclass switch to IgG4 in humans (Meiler et al., 2008). IL-10 was described to inhibit the FcϵRI expression on MCs and basophils thus limiting both their initiate and effector functions (Gillespie et al., 2004). Although there is no direct proof on suppression of FcϵRI by AvCystatin in the grass pollen-induced model, increased production of IL-10 possibly favors not only decreased maintenance and proliferation but also reduced activation of MCs as discussed above. Hence, it would be interesting to further investigate whether AvCystatin acts via suppressed expression of Fc receptor on MCs and basophils.

These modes of action may be approached in further studies and could explain an interference of AvCystatin with MCs and its contribution to diminished airway remodeling and inflammation in the grass pollen-induced model.

AvCystatin induces IL-10 production and $CD4^+CD25^+Foxp3^+$ T cells

Further, AvCystatin led to significantly increased IL-10 levels and total numbers of $CD4^+CD25^+Foxp3^+$ T cells in the spleen. Work from our group on AvCystatin and Ov17 from the human filarial nematode *O. volvulus* showed that both molecules have immunomodulatory capacities and induce secretion of IL-10 in mouse splenocytes or human PBMCs, respectively (Hartmann et al., 1997; Schönemeyer et al., 2001). The immunomodulatory effect was restricted to parasitic cystatins only (Schierack et al., 2003). The effect of Ov17 was mediated by monocytes and was IL-10-dependent. Neutralization of IL-10 restored the expression of costimulatory molecules, and further depletion of monocytes from the PBMC cultures reversed the effects of Ov17 *in vitro* (Schönemeyer et al., 2001). Finally in the previous study when AvCystatin was administered i.p. during the sensitization phase as well as before the challenge with OVA, it effectively ameliorated allergic disease in an IL-10-dependent manner. Blocking of IL-10 with an anti-IL-10R mAb reversed the beneficial effect on cell recruitment and production of IgE. Treatment with AvCystatin significantly increased numbers of $CD4^+CD25^+$ Treg in the peribronchial lymph nodes. Apparently, Treg cells were not the main source of IL-10 as anti-CD25 treatment did not lead to reduction of IL-10 levels. However, depletion of macrophages with clodronate liposomes before airway allergen challenge diminished the anti-allergic effect of AvCystatin, thus pointing into macrophages and IL-10 as main players in this system (Schnoeller et al., 2008). In a recent study, Klotz et al. (2011b) showed that AvCystatin upon uptake by macrophages induces phosphorylation of the mitogen-activated protein kinase signaling pathways ERK1/2 and p38 and IL-10 production. Such data encourages to speculate that IL-10 plays a central role in the immunomodulatory effect of AvCystatin in the grass pollen-induced model of asthma.

Although IL-10 was originally described as a unique product of Th2 clones (Fiorentino et al., 1989), it is a cytokine with broad immunoregulatory function secreted by Th2 lymphocytes themselves, Th1 cells, macrophages, DCs, B lymphocytes, MCs, eosinophils and different regulatory $CD4^+$ T subsets (reviewed in Saraiva and O'Garra, 2010). In the context of asthma, IL-10 inhibits eosinophilia, by suppression of IL-5 and GM-CSF, effecting in eosinophil apoptosis (reviewed in Ogawa et al., 2008).

Furthermore, human immune responses to insect venoms show that successful allergen-immunotherapy is associated with induction of allergen-induced IL-10-secreting T cells, which results in changed balance of IgE and human IgG4 antibodies as well as decreased allergen-specific Th2 responses (Akdis et al., 1998; Akdis and Akdis, 2011). However, in this context different CD4 T cell subsets, as a part of their

regulatory function are capable to secrete IL-10. These are: natural and inducible Foxp3$^+$ Treg as well as Tr1 cells (Lloyd and Hawrylowicz, 2009).

Fortunately in frame of allergic disorders, numerous animal models help to understand the complex role of IL-10. In that way, sensitized and OVA-exposed IL-10-deficient mice were reported for enhanced airway inflammation in comparison to allergic WT controls (Tournoy et al., 2000). Furthermore, *in vivo* IL-10 gene delivery suppressed airway inflammation, AHR and downregulated APC functions in the lung (Nakagome et al., 2005). Thus, induction of IL-10 synthesis is associated with amelioration of disease symptoms. Therefore, AvCystatin-induced levels of IL-10 may drive or directly act on the inhibition of inflammatory cell influx and downregulation of Th2 cytokines. These observations fit the contexts, especially that AvCystatin is derived from a parasitic worm.

Other authors showed that infections with helminths such as *S. mansoni* (Mangan et al., 2006) and *H. polygyrus* (Bashir et al., 2002; Kitagaki et al., 2006) decreased allergen-induced inflammation and the effect was observed in the presence of increased levels of IL-10 and very often elevated numbers of CD4$^+$CD25$^+$Foxp3$^+$ T cells. Moreover, B cells can also serve as a source of IL-10 (Mangan et al., 2004; Amu et al., 2010) In the context of discussed regulatory effect of helminths in asthma, AvCystatin has more advantages because as a single filarial molecule with therapeutic potential acts as a selective immunomodulator. There are only a few examples of individual helminth-derived proteins described (reviewed in Table 1.1, Subsection 1.3.2) that have immunomodulatory capacity in allergy. Interestingly, most of them act via IL-10-, TGF-β-, IFN-γ- and/or CD4$^+$CD25$^+$ T cell-dependent mechanism (reviewed in Daniłowicz-Luebert et al., 2011). As in the present study, treatment with AvCystatin in the grass pollen-induced mouse model of asthma induced high levels of IL-10 and increased numbers of CD4$^+$CD25$^+$Foxp3$^+$ T cells, it would be appealing to further check whether any other cell population (e.g. macrophages, B lymphocytes or DCs) serves as a source of AvCystatin-induced IL-10 in this model.

Although AvCystatin induced high levels of IL-10, asthmatic but not AvCystatin-treated mice were also capable to produce high levels of IL-10 in response to the grass pollen allergen. Thus, it is not yet clear if IL-10 directly mediates the effects of AvCystatin and if so how AvCystatin-specific IL-10 mediates its outcome when high levels of grass pollen-specific IL-10 are also detected in allergic disease controls. This striking fact points into pleiotropic characteristics of this cytokine. And although IL-10 has regulatory capacities (discussed in Romagnani, 2004), some studies indicate that IL-10 rather promotes than regulates airway responsiveness in the allergic asthmatic state. Thus, the role of IL-10 in asthma remains controversial. Robinson et al. (1996) as well as Colavita et al. (2000) reported significantly higher expression of IL-10 mRNA in asthmatic compared with healthy control subjects. Moreover,

IL-10 was shown to augment airway smooth muscle responsiveness and to upregulate mRNA expression and release of IL-5 from cultured asthmatic serum-sensitized airway smooth muscle cells. Thus, there is an association between induced upregulated IL-10 and IL-5 expression. Therefore, the release of IL-5 could be dependent on the induction of IL-10 in the sensitized cell (Grunstein et al., 2001). On the one hand, in our model IL-10 with inflammatory capacities may play a role in the development of asthma by acting directly on the sensitized airway smooth muscle and by promoting expression of IL-5 in asthmatic animals. And on the other side, AvCystatin-induced regulatory IL-10 should immediately prevent sensitization with the grass pollen allergen, which indeed resulted in low levels of IL-5 in this work.

Further, Makela et al. (2000) reported that Th2-type cytokine IL-10 is necessary for the development of AHR but not for airway inflammation or eosinophilia after allergen challenge in already sensitized mice. van Scott et al. (2000) confirmed that IL-10, despite anti-inflammatory activity, augments AHR in ragweed-induced allergy. A recent report shows that IL-10$^+$ Th2 inhibitory (Th2i) cells can originate from non-suppressive Th2 effector precursors under action of effector cytokines such as IL-2 and IL-21. This sheds some light on how Th2 responses may prevent further immunopathologies (Altin et al., 2012). However, it remains unknown how the balance of immunostimulatory and suppressive actions of IL-10 is coordinated during Th2 response induced by an allergen. Thus, a possibility exists that either AvCystatin directly drives the conversion of pathogenic Th2 cells into immunosuppressive IL-10$^+$ Th2 (Th2i) cells in an allergic setup or that upon AvCystatin's treatment IL-10 'switches' its cytokine-modulatory effects in a cell type-specific manner. Also one possibility is that the source of AvCystatin-induced IL-10 is critical. In the present study AvCystatin additionally increased the frequency of regulatory T cells in treated animals, although it is not known whether these cells produced IL-10. Possibly the induction of Tregs or alternative cell sources of IL-10 in AvCystatin-treated mice may actively suppress the pathogenic Th2 cell pool, however this might be addressed in further studies.

The route of AvCystatin's administration

It is important to evaluate treatment regiments that raise effective levels of drug concentration *in vivo* and are convenient for administration for physicians as well as patients themselves. In frame of this work, two parenteral routes of AvCystatin administration in the mouse model of allergy were compared: intraperitoneal (i.p.) and subcutaneous (s.c.).

The route of administration in principle should allow drugs to dissolve in body fluids and preferably enter the circulatory system before they can be distributed to sites of action. Intraperitoneal administration is the injection of a substance into

the peritoneal cavity and due to its ease of administration compared with other parenteral methods in animals, it is rather applied to animals than humans. Moreover any irritating compound, is less irritating if administered i.p. In humans, some chemotherapy drugs (i.e., for treatment of ovarian cancer) are administered via i.p. injections, however there are some controversies about this method (Swart et al., 2008). Intraperitoneally delivered drugs drain into the thoracic lymphatic and the *vena cava*, and are exposed to a larger absorptive surface area than s.c. regimens. On the other hand, s.c. is delivered as a bolus into the subcutis (directly below the dermis and epidermis) and is relatively easy to self-administer. Subcutaneous injections are highly effective, fast (the drug enters the blood after 3-5 minutes), however this may depend on drug solubility and are usually applied for administering vaccines and medications such as insulin, morphine etc., or allergens in subcutaneous immunotherapy. Subcutaneously administered drugs quickly drain to the lymph nodes closest to the injected site where the drug becomes concentrated.

Unfortunately, there are very few studies on animal models examining differences in the routes of drug administration. An extensive search of published reports suggests that the present work is possibly one of very few investigations done on the effect of administration routes of a biological molecule in animal models of allergy. In this study AvCystatin (in PBS as a solvent) applied four times i.p. during sensitization with the allergen downregulated all hallmarks of asthma as discussed before in this Section. However, s.c. treatment under the same conditions did not improve the outcome of allergy, all Th2-associated parameters of allergic asthma remained unaltered. Moreover, there was no increase in levels of AvCystatin specific-IL-10. This shows that s.c. injection is an uneffective way of AvCystatin administration in this mouse model of allergy. As substances injected s.c. enter the blood very fast, it might be that AvCystatin requires a longer time to be present in the cavity, slower migration to lymph nodes and adsorption by the vascular system. AvCystatin optimally acts in the peritoneum but not in the subcutis, probably due to the fact that both sites are said to have different pattern of absorption and excretion, and drug distribution depends also on its molecular weight and solubility. Therefore, one explanation could be that AvCystatin administered s.c. is directed rapidly into the blood circulation and thus is not available to the right cell population. Apparently AvCystatin is capable to exert immunomodulatory capacity efficiently on peritoneal exudate cells (PECs) i.e., macrophages. The peritoneal surface is described as a source of monocytes and anti-inflammatory peritoneal exudate cells (macrophages), thus it has been long time ago proposed as another site to administer tumor or viral vaccines, as well as immunomodulators (Regelson and Parker, 1986).

In the previous work, the tissue distribution of AvCystatin was studied after a single i.v. or i.p. injection of ^{125}I-labelled AvCystatin to healthy BALB/c mice.

^{125}I-labelled AvCystatin within 30 min when injected i.v. showed distribution into the sera, gut, skin, kidneys, liver and finally urine. Whereas, after 30 min of i.p. injections it was found particularly in the gut and body. Three hours after i.p./i.v. administration, AvCystatin was still detected in the gut, skin, body, partially in liver and to the major extend in urine. AvCystatin could not be detected at any time in PBMCs, PBLNs, MLNs, spleen or bladder. Moreover, the half-life of AvCystatin in the animal body was 70 min and it was totally cleared away after 20 hours (Hoffmann 2009, unpublished). This points into various tissue distribution of AvCystatin after different ways of administration. Moreover, AvCystatin is available for rather short time *in vivo*, possibly after s.c. administration as well. Thus, the second justification would be that due to its short-half life and availability in the body as well as probably particular solubility when administered s.c., AvCystatin fails to be distributed to the organs where it could prime the target cells. Papamatheakis et al. (1978) showed ^{14}C-labelled pyran was uptaken by peritoneal macrophages after i.p. injection. Pyran (MVE) activates macrophages that constitute the primary mechanism of pyran-induced host resistance to microbial infection. As AvCystatin was shown to be taken up by macrophages (Klotz et al., 2011b) it would be interesting to assess which organ or cell-compartment is the primary source of such AvCystatin-targeted macrophages and which way of AvCystatin administration activates these cells in the most efficient way.

More work on ways of drugs administration is published for cancer treatment. Earlier pre-clinical and clinical studies on MVE-2, a macrophage activating, interferon inducing anti-cancer component, showed that the site of injection is very crucial. Translation of i.p. data from laboratory animals to i.v. injections in human patients failed. As i.p. site differs in absorption and clearance, drug formulations for i.v. injections in the clinical trials should have been made in different way than for i.p. administration (Wiliam, 1986). Therefore, when thinking about translating the i.p. pre-clinical animal data on AvCystatin to clinical patients, it seems interesting to propose a usage of any stabilizing agent e.g., beads, liposomes or microparticles as alternative ways of AvCystatin i.p. administration to patients. Microparticles were described to retain longer in the peritoneal cavity without the need for frequent drug administration (Kohane et al., 2006). Anti-cancer drug treatment called DTX formed with a polymer-lipid depot delivered i.p. was shown to have good efficacy also on the systemic level suppressing distal metastatic disease sites located outside of the peritoneal cavity (Zahedi et al., 2012). This supports our own data where AvCystatin i.p. had immunomodulatory capacity on the local as well as the systemic level in a stark contrast to s.c. administration. In contrast to the present study, Hough et al. (1996) reported better effects and distribution after s.c. than after i.p. administration of ibogaine, a substance for an anti-addictive treatment. Authors

suggested that i.p. delivered drug undergoes hepatic clearance, and after s.c. delivery some of ibogaine metabolites still remain and account for its activity. In this context, what the metabolites of AvCystatin are and whether any toxicity disabling immunosuppressive effect of the molecule when applied s.c. is exerted, these could be addressed in further studies.

Interestingly AvCystatin s.c.-administered animals showed a trend of decreased MCs numbers in the lungs and MCs activity in the serum (reduced levels of MC-protease). AvCystatin injected s.c. although, lack of suppressive capacity on Th2-induced allergy, strikingly had partial influence on MCs. MCs are also located in the skin tissue as well as mucosa. This might suggest s.c. injections target a distinct mechanism leading to interference with local MC-proliferation and systemic degranulation, independently from Th2-reducing capacity. It was described for omalizumab, that apart from binding free IgEs, it significantly reduced basophil mediator release and decease FcϵRI expression in skin MCs of allergic subjects (Beck et al., 2004). Theoretically, AvCystatin administered either way could cross-link, bind to or suppress activation of Fc receptors on MCs, which was discussed earlier in this Section.

Of a clinical interest as treatment for asthma, it would be interesting to evaluate immunomodulatory capacities of nebulized AvCystatin (inhaled application), which was described to be a very efficient way of therapy in case of treatment of asthma and other diseases (reviewed in Siekmeier and Scheuch, 2008; Rubin, 2010).

4.3 Influence of AvCystatin's characteristics on mediated immunomodulation

When thinking about any specific features of AvCystatin that may play a role in its immunomodulatory capacity, the literature and our own study bring three main factors into consideration: (1) protease inhibitory function, (2) structural characteristics of the molecule, (3) potential posttranslational modifications.

Cysteine protease inhibitory function

First of all, recombinant AvCystatin, expressed in *E. coli*, was described to exhibit biological activity as a cysteine protease inhibitor (Hartmann et al., 1997) and showed high homology to other nematode, arthropod as well as vertebrate cystatins (reviewed in Klotz et al., 2011a). In the present study, amino acid mutations of three conserved domains that constitute cysteine protease-cystatin interaction site and direct the cystatin molecule into the active cleft of the protease, led to a complete loss of biological activity as a protease inhibitor. Mutated AvCystatin (AvCystatin$_{mut}$)

was not able to inhibit protease in the standard papain-inhibition test.

The inhibitory activity is essential for parasitic organisms to regulate their normal biological and physiological processes. A cystatin from human filarial nematode *O. volvulus* was suggested to regulate parasite proteases and lead to cuticle loss during molting and in the development of microfilariae due to its activity as an inhibitor (Lustigman et al., 1992). Analyses of *Caenorhabditis elegans* cystatin CPI-2a pointed into its role during oogenesis and fertilization (Hashmi et al., 2006). Other parasitic cystatins were described to support alimentary needs of *S. mansoni* (Wasilewski et al., 1996) or some ticks by either allowing successful blood meal protecting from host's proteases (Karim et al., 2005) or preventing endogenous peptidases to digest stored blood (Grunclová et al., 2006). On the other hand protease inhibitory activity of cystatins was described to contribute to interference with host's immune responses e.g., the antigen processing and presentation pathway. Schönemeyer et al. (2001) reported that *O. volvulus* cystatin inhibited immunologically relevant host cysteine proteases cathepsin L and S (involved in the endosomal antigen processing) and in consequence interfered with antigen presentation of human APCs. Two tick saliva cystatins sialostatin L and L2 inhibited various mammalian cathepsins and contributed to the blood-feeding success of the ticks (Kotsyfakis et al., 2007). Thanks to their inhibitory activity sialostatins were able to downmodulate MHC-II and costimulatory molecule CD86 on DCs and to suppress antigen-specific proliferation *in vitro* and *in vivo* (Kotsyfakis et al., 2006).

Additionally, cysteine protease inhibitors are described to reduce unrelated inflammatory immune responses. Treatment with nippocystatin from *N. brasiliensis* of OVA-sensitized mice decreased allergen-specific spleen cell proliferation, reduced OVA-specific IgE levels and cytokine production. Authors postulated the effect was due to the inhibition of cathepsin B- and L-dependent antigen processing (Dainichi et al., 2001b). Furthermore, active sialostatin L was reported to reduce AHR, OVA-induced lung pathology and inhibit IL-9 production of Th9 cells (Horka et al., 2012). Also in a study from our group AvCystatin as an active inhibitor suppressed OVA-induced allergic responses (Schnoeller et al., 2008). Therefore, it was striking to see in the present work that AvCystatin was equally efficient in inhibiting eosinophil migration into the lungs, in decreasing allergen-specific antibody levels and Th2-associated cytokines in the grass pollen-induced model independently of its protease inhibitor function. Moreover, mutated AvCytatin (no inhibitory activity) was able to induce production of IL-10 in spleen cells, which speaks for rather a IL-10-driven immunomodulation independent of the protease inhibitory activity. In contrary to our work, Layton et al. (2001) reported that the therapeutic effect of the broad-spectrum cysteine proteinase inhibitor E64 on allergic lung inflammation was mediated by membrane-associated cysteine proteases, possibly cathepsin B. And

although, imbalance between cysteine proteases and cystatins has been suggested to contribute to connective tissue remodelling associated with lung inflammatory processes (Serveau-Avesque et al., 2006), in our study, the effect of AvCystatin surely was not mediated via inhibition of host's proteases. Nevertheless, some endogenous human cystatins were described to interfere with the function of many clinically relevant allergens that as proteases cause allergic reactions. Major allergens of HDM that are active cysteine proteases, Der p 1 and Der f 1, enhance IgE production, induce Th2 responses and contribute to allergy and atopic dermatitis (AD) in human (Comoy et al., 1998; Gough et al., 1999; Thomas et al., 2002). Kato et al. (2005) reported that cystatin A produced by keratinocytes constituted the major biochemical skin barrier, which eliminated HDM cysteine proteases activity in AD scenario. However, not all endogenous cystatins seem to be involved in downregulation of allergies. Asthmatic patients were reported for significantly elevated concentrations of cystatin C in serum when compared to controls (Cimerman et al., 2000). This shows that excess of this protease inhibitor could be rather correlated with asthma, thus probably its inhibitor activity function does not play a role at least not in diminishing of the allergy, although to which allergens subjects were sensitized to, authors did not report (Cimerman et al., 2000).

Imbalance between cystatin C and cysteine proteinases, as well as improper function as an inhibitor has been associated with different diseases such as inflammation, cancer, multiple sclerosis, HIV infection, cardiovascular and kidney disfunction, amyloid angiopathy (reviewed in Turk et al., 2008). Bäcklund et al. (2011) reported that deficiency of cystatin C enhanced collagen-induced arthritis and delayed-type hypersensitivity reaction, which was possibly due to affected priming *in vivo* of the immune system and more activated APCs. However, our study provides an evidence that function as an inhibitor is not necessary for AvCystatin's immunomodulation of Th2-driven pathology via interference with host's proteases.

Hypothetically, inhibitory activity of AvCystatin could play a role in case when allergy is induced by allergens with proteolytic functions such as mite (Der p 1, Der f 1, Eur m 1), coackroach (Per a 10), mold (Cur l 1) or fruit (pineapple - Ana c 2, cantaloupe - Cuc m 1, papaya - Car p 1) allergens. Others reported that inhibition of enzymatic activity of such allergens led to milder inflammation and Th2 cytokine responses (Sudha et al., 2009; Tripathi et al., 2009; Cunningham et al., 2012). Recent reports showed that serine-protease from cockroach and cysteine-proteases of HDM and papaya modulate the innate immune response via activation of protease-activated receptor-2 (PAR-2) (Asokananthan et al., 2002; Day et al., 2010; Liang et al., 2012). Clearly, activity of proteases at mucosal surfaces regulates Th2 immune responses. In case that AvCystatin immunomodulated protease-induced allergy, this inhibitor (AvCystatin) would block allergenic proteases or could interact with

receptors expressed on APCs or airway epithelial cells targeted by such proteolytic allergens. However, in the present study animals were sensitized with Phl p 5b, which functions as an RNAse in plants, so the only proteases driving allergy in this case might come from endogenous proteases (i.e. MCs tryptase) that are released after inflammatory events.

Strikingly some active cysteine protease inhibitors may function as allergens as well. When AvCystatin amino acid sequence was blasted with ALLERGOME database (data not shown), two proteins of *A. simplex* (cystatin - Ani s 4 and paramyosin - Ani s 2) as well as domestic cat cystatin - Fel d 3 showed up as top three allergens with high sequence similarity to AvCystatin. Indeed, Ani s 4 and Fel d 3 have been reported as IgE-reactive cystatins with all main conserved motifs that form the complex with a cystein proteinase (Ichikawa et al., 2001; Rodriguez-Mahillo et al., 2008). However, AvCystatin applied i.p. or i.n. did not induce airway hyperreactivity, did not increase IgE production nor significant numbers of inflammatory cells in the lungs. Moreover, AvCystatin in active and also in non-active form, increased secretion of IL-10 in PEC-derived macrophages (Schnoeller 2008, PhD thesis). Therefore although quite surprising, it becomes clear that inhibitory activity does not lead to AvCystatin's allergenictiy nor constitute the main immunosuppressive feature of this molecule.

Structural aspects

Our own unpublished observations showed that recombinant AvCystatin in the solution forms monomers, dimers and oligomers. Further investigation of AvCystatin's primary amino acid sequence revealed an additional N-terminal cysteine, which can form an intermolecular disulfide bridge with another AvCystatin molecule. Indeed, truncation of this additional cysteine residue predisposed AvCystatin to stay mainly as a mixture of monomers. Two independent techniques, Western blot analysis and a biochemical method - gel filtration and RALS confirmed the results. However, a trace of dimers could be observed in N-terminal truncated AvCystatin (rAvCystatin$_{tr}$).

Under physiological conditions cystatins form monomers (Turk et al., 1985; Ekiel et al., 1997; Kotsyfakis et al., 2010). However, a notable feature of cystatins is appearance as dimers or oligomers. Cystatins' dimers can assemble into tetramers and other higher order structures (Sanders et al., 2004; Jenko Kokalj et al., 2007). Determined dimeric structures of human cystatin C (HCC) as well as human cystatin F (HCF) indicate diverse mechanisms of dimer formation. Protein aggregation of HCC, through conformational changes of the β-hairpin loop L1 as three-dimensional (3D) domain swapping leads to dimerization and then to oligomerization and in return to a severe phenotype of pathological amyloidosis in humans (Janowski et al.,

2001). Additionally, it was found that either naturally occurring point mutation in HCC (L68Q) or first-ten-amino acid residue N-terminally truncated HCC amplify formation of a new crystal structure composed of dimers (Janowski et al., 2004). Moreover HCC oligomers fibrilize faster than monomers (Wahlbom et al., 2007), and as suggested, interact via 3D domain swapping, which was also observed under i*n vitro* conditions in chicken cystatin C (Staniforth et al., 2001) and human stefin B (Zerovnik et al., 2007).

Kotsyfakis et al. (2010) also reported dimerization of a cystatin from tick *I. scapularis*, sialostain L, via domain swapping. Analysis by gel filtration chromatography revealed that a small fraction of sialostatin L was dimerizing at high concentrations of around 10 mg/ml (Kotsyfakis et al., 2010). Therefore it is very likely that the visible dimer band on Western blot or a clear dimer peak species in RALS of AvCytstain$_{tr}$ with no free cysteine available, comes from domain swapped dimers. In contrast to Western blot analysis, high concentrations of AvCystatin were used when preparing samples for RALS, thus it is very likely that increased concentration contributes to AvCystatin's domain swapping. However, the most precise answer for domain swapping of AvCystatin would give crystallographic resolution of the structure, not a method in solution, as only crystals can efficiently stabilize the structure, as shown for HCC or sialostatin L (Ekiel et al., 1997; Kotsyfakis et al., 2010).

In HCF dimers are formed by two monomers interacting via an intermolecular disulphide bond (Schüttelkopf et al., 2006). Aggregations can cause complete loss of inhibitory activity (Ekiel and Abrahamson, 1996). Dimers of HCC, HCF and sialostatin L were described to reduce the inhibitory function of cystatin because the protease-binding surfaces of the cystatin were disturbed (Abrahamson and Grubb, 1994; Wei et al., 1998; Langerholc et al., 2005; Kotsyfakis et al., 2010). In contrast to that, although clear oligomerization is present, AvCystatin remains active as cysteine protease inhibitor. This suggests that despite conformational alterations resulting from oligomerization, the protease binding sites are still accessible to proteases. However, this does not exclude the possibility that aggregation may affect conformation of other domains, which possibly account for the overall immunomodulatory character of AvCystatin.

Additionally, in this study treatment of grass pollen allergic mice with either recombinant AvCystatin's derivate that is not active as protease inhibitor or with its monomeric version showed comparable immunosuppressive effect as unmodified recombinant AvCystatin, which was used in previous studies in the OVA-induced model of asthma (Schnoeller et al., 2008). Concerning oligomerization, interestingly Yang et al. (1993) proposed that high 'density' of antigen can modify immune responses possibly due to minimal antigen presentation of the oligomeric form. An oligomeric form of antigen induced a different profile of T cell cytokines, than that caused by a natural monomeric version (Yang et al., 1993). However, in the present

study the effect of AvCystatin immunomodulatory capacity in an asthma model was independent of oligomerization via N-terminal disulfide bond. Concerning an eventual involvement of PTMs in the immunomodulatory effect of AvCystatin, apart from performed here an *in vitro* proliferation assay, more studies on the confirmation of an obtained modification by e.g., mass-spectrometry analysis, and validation of demethylated recombinant AvCystatin (rAvCystatin$_{dm}$) in an *in vivo* model of allergic asthma could be addressed in future studies.

4.4 AvCystatin and allergen-specific immunotherapy

Allergen-specific immunotherapy is the most promising so far existing treatment for allergy. Data discussed above and published work demonstrated that AvCystatin might be a promising candidate potentiating regulatory mechanisms in the allergen-specific immunotherapy. Thus, in frame of this thesis based on the OVA-induced mouse model for allergic asthma, a pre-clinical model of OVA-induced imunotherapy (SIT) in mice was established in order to test an adjuvant effect of AvCystatin.

OVA-sensitized and challenged animals showed all clinical symptoms of allergy, including airway eosinophilia, increased Th2-cytokine production in the BAL fluid and serum levels of total IgE, OVA-specific IgE and IgG1 antibodies. Subcutaneously treated mice with three, either low or high, doses of OVA (SIT therapy) showed an abrogation in nearly all allergic parameters, such as reduced inflammatory cell migration into the lungs, decreased production of IL-5 and IL-13 and allergen-specific IgEs. Thus, the protocol applied in the present work mimics beneficial results from immunotherapy in humans. Similarly, van Oosterhout et al. (1998) reported that increasing doses of OVA led to reduced eosinophil influx into the BAL fluid, lower levels of IL-4 and decreased production of OVA-specific IgE in their newly established mouse model. However, unlike in our study, van Oosterhout et al. observed also an increase in OVA-specific IgG2a antibodies. van Oosterhout et al. used a protocol of 8-week treatment with increasing doses of OVA (van Oosterhout et al., 1998). In our study, animals received SIT high or low dose for four days before allergen-challenge. Moreover, in the present work application of SIT high dose increased IL-10 production in the BAL and levels of allergen-specific IgG1 (as IgG4 in human) that constitute immunemodifying mechanisms of specific immunotherapy. Interestingly, only immunotherapy with entire OVA, but not with the immunodominant OVA epitope, was shown to have beneficial effects on downmodulation of disease symptoms in a mouse model of allergic asthma (Janssen et al., 1999). Currently Treg cells that produce the immunosuppressive cytokines IL-10 and TGF-β are thought to be mostly responsible for clinically successful SIT (reviewed in Akdis and Akdis, 2009).

Thus, considering above, investigation of refined strategies for immunotherapy that facilitate the induction of regulatory mechanisms e.g., using improved and/or alternative adjuvants in combination with SIT would raise safety and increase efficacy of SIT (Kopp, 2011).

Adjuvant capacity

In the present study, the combination of AvCystatin with SIT resulted in the opposite effects than hypothesized. Cystatin either did not have any boosting effect or it deteriorated the beneficial outcome of SIT. AvCystatin mixed and delivered s.c. with SIT, or administered s.c./i.p. separately from SIT did not ameliorate OVA-induced allergy. All applications of AvCystatin together with allergen s.c. led to recall Th2 cytokine production in response to AvCystatin *in vitro*. No substantial IL-10 induction was observed, except when AvCystatin was mixed and injected s.c. together with OVA. Strikingly, when AvCystatin was delivered i.p. and subsequently with SIT, it rather enhanced total cell and eosinophil infiltration into the lungs, as well as increased levels of OVA-specific IgG1, although IL-4 levels were decreased. This suggests divergent and independent mechanisms of action of the allergen and AvCystatin when it is applied after a sensitization phase along with the allergen.

In the context of AvCystatin as an adjuvant, the timing and route of administration plays an important role. It becomes clear that in general AvCystatin s.c. injections during allergen sensitization do not elicit desired immunoregulatory mechanisms as it was observed in the model with the clinically relevant grass pollen allergen (discussed in Section 4.2). In another study, the beneficial effect was observed for glucocorticoid and vitamin D3 as adjuvants to SIT when the drugs were given at the same time and route as the allergen (Taher et al., 2008). In immunotherapy the antigen composition and route of administration of the allergen determine the effectiveness of SIT treatment. Subcutaneous immunotherapy with OVA but not i.n. had beneficial effects. Interestingly, s.c. application of OVA peptide deteriorated allergy symptoms (Janssen et al., 2000). It remains of interest to determine which mode of delivery can elicit a boosting mechanism of AvCystatin as an adjuvant that is required for suppression of Th2-cell activity.

There is more evidence that alternative delivery of allergens coupled to carbohydrate or virus-like particles or administered as fusion proteins gives promising results. Carbohydrate based particles (CBP)-bound rPhl p 5b administered s.c. as immunotherapy was shown to decrease allergen-specific IgE levels, to induce mixed allergen-specific Th1 immune responses detected as elevated production of IFN-γ and allergen-specific IgG2a antibodies, and in contrast to a standard adjuvant - Alum, did not cause granuloma formation at the injection sites. Moreover, CBPs mi-

xed with rPhl p 5b (but not coupled to the allergen) promoted strong Th2 responses (Grönlund et al., 2002). Antigens joined with CBPs are more effectively processed and presented by the APCs than uncoupled antigens (Kovacsovics-Bankowski et al., 1993). In this context, macrophages were described to phagocyte various types of larger particles (Geiser, 2002). Moreover, Andersson et al. (2004) showed that recombinant cat allergen (rFel d 1) coupled with CPBs efficiently activated human DCs *in vitro*. Coupled particles rFel d 1-CBP were uptaken by DCs and unlike free rFel d 1, the complexes upregulated expression of the costimulatory molecule CD86 on DCs surface. In a more recent study, improved therapeutic efficacy and reduced reactogenicity of rFel d 1 was shown by coupling to a repetitive structure of virus-like particles (VLPs). Application of rFel d 1-VLP s.c. protected mice from systemic anaphylaxis, inhibited local MCs degranulation and prevented IgE memory responses after challenge with an allergen. The effect of allergen-particle complexes was mediated by rFel d 1-specific serum IgGs (Schmitz et al., 2009). Although not described in this model so far, it is appealing to speculate that CPB/particle-AvCystatin coupled with an allergen would display a boosting effect without alteration of its immunological properties. Because of the particulate nature of the CBPs such complexes would possibly be presented to proper APCs inducing desired T cell responses.

Another possibility to achieve determined immunomodulatory boosting effects would be creating a fusion protein - rAvCystatin:allergen. Recently, Schülke et al. (2011) provided a strong evidence that the fusion of a recombinant flagellinA, the Toll-like receptor (TLR) 5 agonist, with OVA (rflaA:OVA) enhanced suppression of IL-4 and IL-5 from naive and Th2-biased $CD4^+$ T cells and reduced OVA-induced intestinal allergy *in vivo*. The effect was mediated by IL-10. The fusion protein rflaA:OVA but not equimolar amounts of rflaA or OVA administered alone or as a mixture induced OVA-specific IgG2a while reducing levels of OVA-specific IgE. Similarly to the present study, administration of a mixture OVA plus flagellinA deteriorated OVA-specific IgE levels. It was proposed that the fusion protein facilitates targeting of effector cells. In an earlier study another group reported that the major birch allergen Bet v 1 fused with the bacterial cell surface (S-layer) protein (rSbsC-Bet v 1) induced production of IFN-γ together with IL-10, but no Th2-like responses in cells of birch allergic patients. It was noted that Bet v 1 allergen was correctly folded with all relevant Bet v 1-specific B and T cell epitopes, however with reduced capacity to induce histamine release (Bohle et al., 2004).

Above discussed components have intrinsic Th1-enhancing properties and target TLRs. Despite the unknown receptor-triggered mechanism by AvCystatin, it would be interesting to study recombinantly fused AvCystatin with the allergen. Although not addressed in the present work, such fusion could allow the molecule to directly target antigen-specific cells and possibly instead of worsening the effect of SIT, AvCy-

statin could enhance its suppressive capacity targeting the effector cells. Moreover, one of the advantages of a rAvCystatin:allergen fusion protein would be the constant ratio between allergen and adjuvant (AvCystatin). This would allow standardization and prevent variability of immunological responses *in vivo*. Additionally, the allergen and the parasitic immunomodulator could be delivered to the same APCs. However, in such approaches, considering all results from the present study, schedule of an application route, dosage and frequency of CPB-AvCystatin-allergen particles or a rAvCystatin:allergen fusion protein should be carefully evaluated. Furthermore, an approach of direct expression of a fusion S-layer protein with AvCystatin and the clinically relevant allergen (i.e., rPhl p 5) could be applied, as recently showed by a production of a pyrogen-free fusion S-layer protein with Bet v 1 allergen in the non-pathogenic transformed *Bacillus subtilis* cells. The fusion protein was secreted directly to the culture medium giving self-assembly functional products (Ilk et al., 2011). Another method employing engineered bacterial cells was used by Li et al. (2003). Rectal but not s.c. administration of heat-inactivated producing peanut allergen *E. coli* decreased Th2 cytokine production while increasing IFN-γ and TGF-β in a murine model of peanut allergy (Li et al., 2003). Thus, it would be interesting to evaluate engineered systems to produce AvCystatin with a function as an adjuvant for SIT.

In the course of this work on SIT, the question emerged whether AvCystatin applied alone can interfere with this rather strong OVA-induced model. Hence, when AvCystatin was applied i.p. after the sensitization phase but before challenge, reduced AHR, significantly decreased eosiophilia and levels of allergen-specific Th2 cytokines, suppressed total and OVA-specific IgE in the serum were observed. This proves that AvCystatin was in general capable to suppress the model and elicit its immunomodualtory effect. An increase in AvCystatin-specific IL-10 was observed. AvCystatin applied i.p. induced only production of IL-10 when cells where restimulated with the molecule *in vitro*. That was in contrast to other 'AvCystatin and/plus SIT' approaches that induced also production of Th2-associated cytokines after AvCystatin-restimulation. AvCystatin delivered i.p. but also s.c. reduced goblet cell hyperplasia.

Data obtained in this work suggest that AvCystatin itself has immunomodulatory capacity in the strong OVA-induced allergy model. However, when it is applied along with the allergen (mixed and/or injected at the same side), it is not able to elicit beneficial effects. The possibility exists that when applied along with the allergen as two separate molecules, AvCystatin is presented by different APCs than the allergen, thus causes a different activation state in the APCs, which may greatly affect the outcome of the target cell response. Alternatively, the distribution of the protein may differ after s.c. administration, which can result in various T-cell or other

cell (preferably macrophage) activation at different locations. Therefore, approaches discussed above or application of AvCystatin as adjuvant along with the allergen via different routes (e.g., intralymphatic injections, reviewed in Kündig, 2011) could be taken into consideration to advance an adjuvant effect of this parasitic molecule in the SIT treatment.

4.5 AvCystatin and human allergics

In frame of this thesis, the last step towards evaluation of AvCystatin's effect on allergy was a 'translational' model with cells from grass pollen allergic patients.

In the pilot experiment outside of the grass pollen season with three allergic and two healthy controls, induction of significant Th2 allergen-specific responses were detected for allergic subjects. Stimulation with timothy grass pollen extract of allergic PBMCs resulted in increased allergen-specific cell proliferation, the effect was LPS-independent. Moreover, allergen stimulation induced strong production of IL-13.

Furthermore, in line with many studies, levels of allergen-induced IL-4 and IL-5 were difficult to detect in supernatants at day 3 of the cell culture. Possibly, IL-4 and IL-5 cytokines could be secreted at low levels earlier, with peak production as early as 24 - 48h of culture as described by Hough et al. (1996). Interestingly, results from allergic PBMCs stimulated with a HDM allergen showed peak of IL-4 production before that of IL-5 and IFN-γ, with a rapid decline in the time. However, increase of IL-5, IL-13 and IFN-γ was present in a time-dependent manner until day 7 (Wakugawa et al., 2001). Other studies also report on difficulties with IL-4 detection after antigen stimulation, due to low levels of this cytokine in the cell culture supernatants (Looney et al., 1994; Wurtzen et al., 1998). However in some studies it was possible to measure early allergen-induced IL-4 production in minute amounts of the femtogram per milliliter range (Kimura et al., 1998).

The pilot experiment revealed that pre-incubation with AvCystatin before allergen stimulation led to a significant decrease of grass pollen-specific IL-13 by PBMCs from allergic patients and resulted in increased levels of IL-10 and a significant production of IFN-γ by allergen-specific $CD4^+$ T cells. Furthermore, increased production of IFN-γ by responding allergic $CD4^+$ T cells was confirmed via flow cytometry. In the same line to our observations, Park et al. (2012) recently reported that stimulation with the recombinant MIF homologue of *A. simplex* (rAs-MIF) increased levels of IFN-γ and IL-10, while decreasing Th2-cytokines in PBMCs from atopic asthmatics but not from patients with non-atopic asthma (Park et al., 2012). However, in this study patients' PBMCs were only stimulated polyclonally with mitogen and not in

an allergen-specific manner as in the present study. Our and their work show that a parasitic molecule may induce IFN-γ, which can be involved in the immunomodulation of human PBMCs from allergic patients.

Having the system established, the capacity of AvCystatin was evaluated in the pollen season on PBMCs from 21 allergic subjects. In the studied group of allergic patients it was possible to induce Th2 immune responses that were characterized by significant increase of allergen-specific IL-13, IL-10 and proliferating $CD4^+IL\text{-}4^+$ T cells. A statistical analysis with Spearman's test revealed a significant correlation between frequencies of proliferating allergen-specific $CD4^+IL\text{-}4^+$ T cells and allergen-specific IgE levels. This means that the stronger the allergy, the higher precursor frequency or the better $CD4^+$ T cell were responding to the allergen. Nurse et al. (2000) also observed positive correlation between allergen-stimulated proliferation of PBMCs and allergen-specific IgE levels it their study. Moreover, a positive association between Th2 cytokine production and proliferation was reported pointing into the fact that the allergen provoked Th2-type responses in cells that specifically recognize the allergen (Nurse et al., 2000). Additionally, Machura et al. (2010) showed that the severity of asthma was associated with increased IgE levels.

In an allergic state certain proportions of Th1 and Th2-type cytokines are produced by allergen-specific T cell clones. It has been proposed that the overall imbalance in Th2 to Th1 cytokine ratios rather than absolute amounts of individual cytokines is responsible for the development of allergy (Tournoy et al., 2002; Romagnani, 2004; Ngoc et al., 2005). Some studies analyzed amount of Th2-type allergen-stimulated cytokine produced in relation to levels of IFN-γ, the major Th1 cytokine. Presence of AvCystatin, in relation to Th2 cytokine production, resulted in a significant conversion from a Th2- to a Th1-biased response. The Th2/Th1 cytokine ratio was calculated based on IL-13 to IFN-γ ratio in stimulated PBMCs and was decreased after treatment with AvCystatin. This can be explained by the fact that no significant change in IL-13 production was observed after *in vitro* application of AvCystatin prior to GPE-stimulation, but there was a notable increase in IFN-γ. Thus, Th2/Th1 ratio was substantially decreased.

Furthermore, a reduction of proliferating $CD4^+IL\text{-}4^+$ T cells and increased frequencies of proliferating $CD4^+IFN\text{-}\gamma^+$ T cells were detected after stimulation with AvCystatin. This suggests that AvCystatin is able to downregulate IL-4 cytokine responses of allergen-specific $CD4^+$ T cells, which goes in line with induced levels of IFN-γ that possibly generate the inhibitory effect on allergen-reactive $CD4^+$IL-4 producing T cells.

With regard to allergy treatment many studies have reported that IFN-γ seems to be beneficial and is detected in patients after successful allergen-specific immu-

notherapy (Larche et al., 2006). Immunotherapy in atopic patients induces allergen-specific Th1 cells that suppress the effector function of allergen-specific Th2 cells and alter antibody production (Ebner et al., 1997; Akdis and Akdis, 2011). IFN-γ is known to be present in healthy subjects but not in atopic patients when assessing levels of this cytokine produced *in vivo* (Hough et al., 1996; Holgate and Polosa, 2008). Leonard et al. (1997) proposed that the difference between atopy and asthma is not related to high production of Th2 cytokines (i.e., IL-4), but may be inversely correlated with levels of IFN-γ in response to HDM stimulation of allergic PBMCs (Leonard et al., 1997) . Similarly another study demonstrated a selective redirection of ragweed-specific Th2 responses towards Th1 responses with a significant increase of IFN-γ production (Simons et al., 2004). However, the exact role of IFN-γ in regulating allergic disease remains controversial. Nevertheless, there are also numerous studies reporting a significant negative correlation between levels of Th2-associated cytokines and IFN-γ as promising allergy treatment in the human system (Pochard et al., 2002). A defective production of IFN-γ after *in vitro* restimulation of PBMCs constitutes a general feature of atopic disease (Machura et al., 2010). IFN-γ can directly inhibit the production of Th2 cytokines in a bystander manner and thus, in this fashion may mediate decreases in Th2 associated changes that lead to allergic disease including the production of allergen-specific IgE (Pène et al., 1988; Romagnani, 1990; Parronchi et al., 1992). Of interest, in the frame of this thesis neither in the animal model of grass pollen-induced allergy nor in the SIT model any substantial induction of IFN-γ in AvCystatin-treated animals was observed. Additionally, upon *in vitro* restimulation of spleen cells from asthmatic animals with AvCystatin prior to the allergen, no IFN-γ production was detected (own unpublished data). This points into a unique effect of AvCystatin elicited *in vitro* on human cells from allergic patients.

Future studies may address following issues: whether upon stimulation with AvCystatin the IFN-γ-producing cells are only restricted to $CD4^+$ T cells, whether AvCystatin-induced IFN-γ^+ cells show suppressive capacity or which mechanism can be observed by blocking AvCystatin-specific IFN-γ.

AvCystatin in a mouse system *in vivo* acts on macrophages (Schnoeller et al., 2008; Klotz et al., 2011b), which results in the production of IL-10. In PBMCs, the source of IL-10 is thought to be the monocyte, dendritic cell, B or T cell (reviewed in Braga et al., 2011; Moldaver and Larche, 2011; Platts-Mills and Woodfolk, 2011). In an early study on monocytes from allergic subjects, exogenous IL-10 significantly increased the proportion of suppressive cells and led to decrease of allogenic mixed lymphocyte proliferation (Tormey et al., 1998). Moreover, a decreased spontaneous and LPS-induced IL-10 production was observed by monocytes from allergic patients (Borish et al., 1996). In the present study in the pollen season, there was no

substantial change in frequencies of allergen-proliferating $CD4^{+}IL\text{-}10^{+}$ T cells after incubation with AvCystatin and subsequently with GPE. One plausible explanation for that could be that in the mixed T cell population, which is the PBMCs culture, AvCystatin cannot efficiently target APCs or T cells to induce IL-10 production. Our observations led to the conclusions that AvCystatin may act on the APCs/T cells via induction of regulatory macrophages that possibly are able to interfere with antigen presentation or interact with T cells (N.L. O'Regan, personal communication). Of note, there are no macrophages present in the PBMCs. Normally, circulating monocytes that originate from progenitors in the bone marrow leave the bloodstream and migrate into tissues where, following conditioning by local growth factors, they differentiate into macrophage or dendritic cell populations. *In vitro* differentiation from human PBMCs-derived monocytes to macrophages can be achieved in the laboratory (reviewed in Shi and Pamer, 2011). Furthermore, work of our group in mice shows that AvCystatin targets murine macrophages and changes their phenotype to regulatory ones. Such regulatory type of macrophages are able to downregulate unrelated inflammation *in vivo* in a mouse system (Ziegler, in preparation). In the present study, the suppressive capacity of AvCystatin in human allergic PBMCs obtained in the pollen season could not be observed. One possibility could be that AvCystatin was not able to act on its target cell, the macrophage to initiate its immunomodulatory actions. Another explanation for that can be that AvCystatin was exposed to already established allergic Th2-biased cells that were present in the PBMCs culture. Thus, AvCystatin was not effective in the presence of effector cells and only a minority of naive T cells. This likely led to inability to alter Th2-established responses. Possibly AvCystatin failed the competition with GPE to modulate APCs or influence T cell responses. PBMCs were derived from patients with already well built allergy. In the grass pollen-induced mouse model, AvCystatin possibly targets naive cells at the very first time of treatment (day 1), whereas in the culture with allergic PBMCs the abundance of naive cells is very low for AvCystatin to dysregulate the Th2 established responses. Thus, it becomes evident that results from treatment with AvCystatin in the murine model of allergy induced by the clinically relevant allergen should be carefully interpreted and translated with caution into the human system *in vitro* although restimulation was done by the same allergen. Apparently the molecule may target distinct mechanisms in both systems.

One explanation of differences between results from the pilot study and the trial with 21 allergic patients may be caused by the timing of both experiments. The first was performed outside of the pollen season, the latter during the season. Differences based on the pollination season in the profile of cytokine production and allergen-specific proliferation by PBMCs from allergic patients were reported by Gagnon

et al. (1993). Natural exposure to pollens in allergic subjects causes the imbalance between Th1 and Th2 cells in peripheral blood of atopic subjects by stimulation and release of Th2 cells into the systemic circulation leading to increased Th2-cytokines production. In addition, allergen-specific memory T cells decrease after the pollen season, but 60% survive up to 8 months after the season (Horiguchi et al., 2008). Outside the pollen season there are relatively low numbers of allergen-reactive Th2 cells in PBMC of allergic patients, which is as low as 1 per 20 000 cells (Gabrielsson et al., 1997). Difference in results may be caused also by the fact that both study groups were exposed to different pollination seasons. It is important to keep in mind that each pollen season differs in the amount of pollen count and other environmental variables that influence immunological responses of individual patients throughout years (Sakaguchi et al., 2001; Andersson and Lidholm, 2003; Horiguchi et al., 2008; Anderson et al., 2009; Puc, 2011). Hence, a possibility exists that AvCystatin's effect depends on the season and that it modulates T cell responses only out of the pollen season, where a lower percentage of memory Th2 cells circulate. As promising data was obtained only from the pilot study out of the season, it would be interesting to perform further detailed timing and dose establishing experiments that take place in different pollination seasons.

More experiments in different times of the year, revealing the target cell and mechanism of AvCystatin's action would give deeper understanding on its immunomodulatory capacity in the human system with allergic patients.

5. Outlook

First of all, the present work reveals early effects of MCs to orchestrate the development of Th2 immunity upon helminth infection (Fig. 5.1, upper panel). Future studies should investigate what mediator is responsible for this effect and which cells are responding to MC-specific signals by early production of cytokines such as IL-25, IL-33 or TSLP.

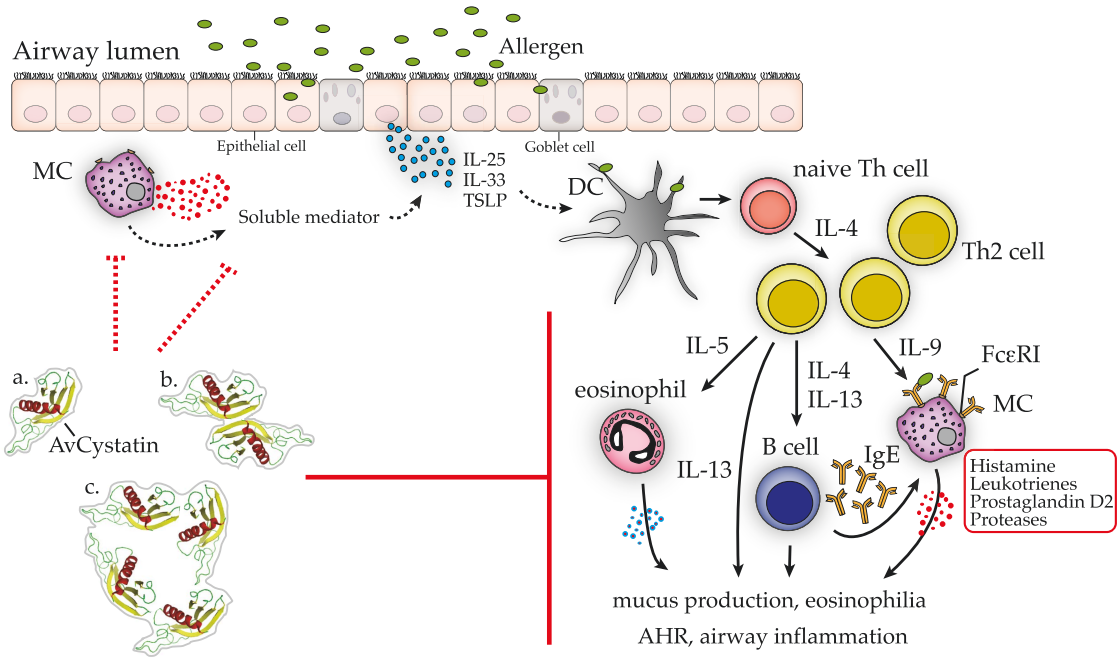

Figure 5.1: Summary of AvCystatin's effect *in vivo* and proposed mechanism to be investigated whether the molecule influences mast cells (MC) to control the development of Th2 immune responses induced by a clinically relevant allergen *in vivo*. AvCystatin (putative 3D structure) independently of protease inhibitory activity, as monomers (a.), dimers (b.) or oligomers (c.), is able to suppress grass pollen-induced allergic reactions. AvCystatin influences actions of MCs, possibly early on during development of Th2-type responses or during the later phases of inflammation via suppression of activation/degranulation of MCs, reduction of the effect of IgE or via cross-linking Fc receptors (see Discussion). Dashed lines indicate proposed mechanism that may be further investigated.

Furthermore, this understanding can be extended and studied during priming events in allergy. Additionally, to exploit MC-suppressory capacity of AvCystatin, further experiments testing the plausible role of this molecule to interfere with MCs and/or their effects *in vitro* as well as in a mouse model of allergy, may be addressed

in the MC-deficient mouse strains (proposed model in Fig. 5.1). Moreover, an *in vitro* system with activated MCs (pre)incubated with AvCystatin can be analyzed to further investigate mechanism of AvCystatin's MC-inhibitory effect.

Furthermore, the present study demonstrates an immunomodulatory capacity of AvCystatin to interfere with a more clinically relevant setting of allergy induced by grass pollen. The effect has been shown to be administration route-specific. Moreover AvCystatin's immunomodulation was independent of its intrinsic features (summarized in Fig. 5.1).

Future work may address other clinical routes of AvCystatin's delivery, such as inhalation aerosol or oral application. Additionaly, applying AvCystatin in the therapeutic setup with a stabilized allergy (so after sensitization and challenge phase) and before a second round of challenges, would closer resemble the situation allergic subjects experience in the pollen season. Moreover, studies on the protein's structure might investigate whether posttranslational modifications, such as methylations play a role in the AvCystatin's modulatory behavior *in vivo*. Furthermore, by crystallization experiments, the possibility of dimerization through domain swapping could be examined. This may shed a light on AvCystatin's crystal-stabilized structure and would be beneficial in case of formulation as a drug in the future.

Another aspect to be tested is the target cell and mechanism of AvCystatin's action *in vitro* on human cells from allergic as well as healthy subjects. This may be done in the cell culture with defined proportions of antigen presenting cells (such as monocytes, DCs but also B cells) and naive/memory T cells incubated with the clinically defined antigen such as grass pollen allergen. In such setup cytokine (i.e., IFN-γ and/or IL-10) depletion experiments could be implemented.

Regarding AvCystatin's capability as an adjuvant in allergen-specific immunotherapy (SIT), future studies may focus on investigation of boosting effects of AvCystatin-coupled-carbohydrate based particles (CBP) or on AvCystatin fused to e.g., clinically relevant grass pollen allergen (rAvCystatin:rPhl p 5b) in the SIT treatment. Such approach may enable to combine the AvCystatin's potent cytokine-modulating activity with reduced allergenicity of grass allergen formulated as a fusion protein with AvCystatin.

6. Methods

6.1 Molecular biology and biochemical methods

6.1.1 Expression and purification of recombinant *Acanthocheilonema viteae* cystatin and derivates

The cDNA of *A. viteae* cystatin (AvCystatin) was cloned in pET28b(+) and expressed in *Escherichia coli* as previously described (Hartmann et al., 1997).

Unmodified recombinant filarial cystatin (rAvCystatin) was purified by affinity chromatography using a Ni-NTA column and dialyzed overnight (O/N), 4°C against PBS/0.05% Triton X-100 or PBS. Next day proteins were dialyzed for additional 2 hours in fresh PBS/0.05% Triton X-100 or PBS. The procedure of expression of recombinant mouse dihydrofolat reductase (rDHFR) was similar to rAvCystatin (Hartmann et al., 1997) but bacterial cultures contained 50 μg/ml kanamycin and 100 μg/ml ampicillin. Further rDHFR was purified by an Imidazol gradient using FPLC (fast performance liquid chromatography) and HiTrap FF columns (similar to Ni-NTA). Dialysis was performed as described above.

6.1.2 Production of mutated and truncated AvCystatin's derivates

Point mutations in the conserved motifs involved in cysteine protease inhibitor activity were introduced (J. Russ and T. Buhrke, unpublished).

Briefly, rAvCystatin$_{mut}$ was obtained by site-directed mutagenesis of three domains to, first of all disrupt the protease inhibition function and at the same time to preserve the overall conformational structure of the molecule. Thus, two leucines and two glycines (LLGG) from N-terminal active domain were exchanged to serine and three alanines (SAAA), from the second conserved motif one glutamine and two valines (QVV) were mutated to serine, alanine and serine (SAS), mutation of tryptophan (W) effected in alanine (A) in C-terminal domain. To this end, rpET28b(+)/AvCystatin was used as a template for the two-step PCR with the high-fidelity Phusion DNA Polymerase (New England Biolabs, MA, USA) and two primer pairs containing mutations. Primers: **Fw1** 5'GAT-

CGAATTCGGTTTTGGTGCGCTGTGAAGAACCCGCAAATATGGAATCT-GAGGTACAAGCGCCCAATTCAGCGGCCGCATGGCAGGAACGCAATC 3' and **Rv1** 5'TATCTCAGGCCTGCGCTAGCGCTAGATGAAACTTTCAGTA 3'; **Fw2** 5'GTGGCAGGCCTGAGATACAAGATG 3' and **Rv2** 5'AGC-TAAGCTTTCACACTGATGAGAGTACTTCTTTTTTTTCCAGAATTTT-GACTTGCAAAAAATTTTCAGCTGATTTCTCCCATGCCTCC 3'.

Obtained products were cloned first into the high-copy number plasmid pSL1180 and DH5α *E. coli* cells were transformed. Then, the entire sequence of mutated rAvCystatin was recloned to pET28b(+), with which BL21(DE3) *E. coli* were transformed. Expression and purification of mutated rAvCystatin (rAvCystatin$_{mut}$) was performed as previously described (Hartmann et al., 1997).

In order to force rAvCystatin to form monomers, the first 15 N-terminal nucleotides (at positions 1-15) were truncated (T. Buhrke, unpublished). A truncated form (rAvCystatin$_{tr}$) was cloned in the same way as unmodified rAvCystatin (Hartmann et al., 1997).

6.1.3 Demethylation, expression and purification of AvCystatin

In order to remove the methylation detected at lysine (K) p.137, a site-directed mutagenesis on rpET28b(+)/AvCystatin using Phusion Kit (Finnzymes, Espoo, Finnland) was performed.

The mutagenesis protocol comprised three steps (Fig. 6.1):

1. PCR amplification of rpET28b(+)/AvCystatin with two phosphorylated primers that were designed in the way to introduce the desired mutation so that they first anneal back to back to the plasmid. Primers: forward containing mutation (underlined) **dmAv17F1** 5'PH-GGCATGGGAGCTGTCATGGG 3' and reversed **dmAv17R3** 5'PH-TCCAACGTGATAATCTGATCCGGATGTCC 3' (TIB, Berlin, Germany). Conditions of the reaction: 2 min at 98°C; 35 cycles: 30 sek at 98°C, 45 sek at Tm=56°C, 1 min 30 sek at 72°C; 10 min at 72°C. To improve yield and specificity 3% DMSO was used in the reaction. In the PCR reaction, Phusion Hot Start DNA Polymerase (New England Biolabs, Frankfurt, Germany) extended the primers and amplified the plasmid with the mutation.

2. Circularization of 6 ng/μl mutated PCR product was done by ligation with Quick T4 DNA Ligase (New England Biolabs, Frankfurt, Germany) for 5 min at room temperature (RT).

3. Transformation of the product to DH5α *E. coli* was performed by incubating cells and the PCR product for 20 min on ice, 1 min at 42°C, further 10 min on ice. SOC medium was added and cells were incubated shaking for 1h at 37°C. Transformed, incubated bacteria were plated into LB-agar plates containing IPTG (1 mM), X-gal (20 mg/ml) and kanamycin (100 μg/ml) for blue (mutated) and white (unmutated) colony differentiation. Efficiency of mutagenesis was evaluated by screening of mutant clones by direct sequencing.

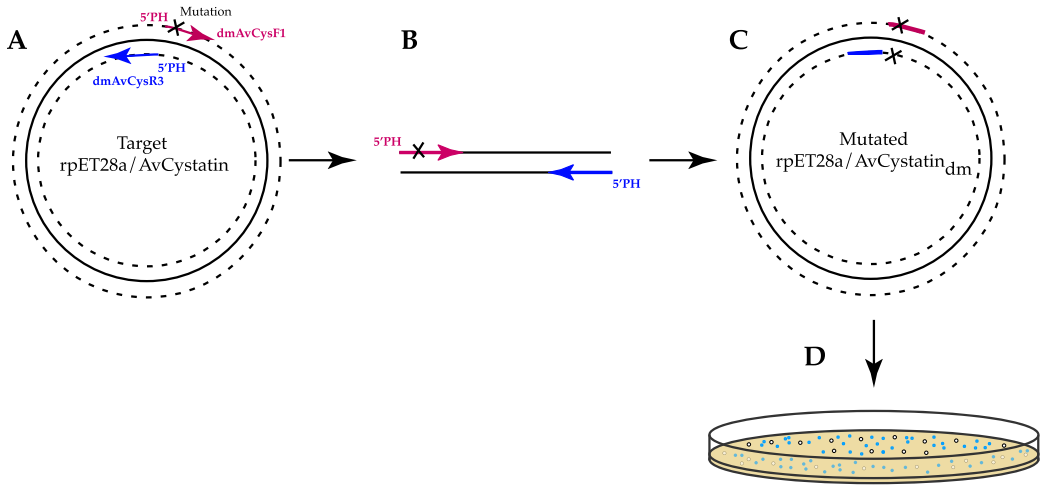

Figure 6.1: Flow chart of site-directed mutaganesis protocol with rAvCystatin. (A) rpET28b(+)/AvCystatin vector was used as a target plasmid and starting material. (B) Phosphorylated primers introduced the desired point mutation c.409_411AAA>CTG. (C) and (D) Mutated target plasmid was amplified, circularized and transformed into bacteria.

In the next step, five blue colonies (clone 1-5) were screened and all contained desired mutation (Fig. 6.1D). Thus, a DNA mutation of three aminoacids (c.409_411AAA>CTG) was achieved without changes in open reading frame (ORF), resulting in amino-acid mutation of lysine (K) to leucine (L) (p.K137L) (Fig. 3.20). Clone 2 was chosen for further experiments. Plasmid DNA was isolated using Plasmid-Mini-Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany). BL21(DE3) *E. coli* were transformed and plated on LB-Agar plates to differentiate as described above. Expression and purification of demethylated rAvCystatin (rAvCystatin$_{dm}$) was performed as previously described (Hartmann et al., 1997).

6.1.4 Endotrap system to remove endotoxin contaminations

EndoTrap blue columns were used according to the manufacturer's instructions (Profos, Regensburg, Germany) to remove endotoxin contaminations in the purified four derivates of rAvCystatin.

6.1.5 Limulus amoebocyte test (endotoxin measurement)

Final endotoxin concentrations were detected by a *Limulus* amoebocyte lysate test from horseshoe crab, *Limulus polyphemus* (Cambrex, BioSciences, Walkersville, USA). Average endotoxin concentration of rAvCystatin, rAvCystatin$_{mut}$, rAvCystatin$_{tr}$ and rAvCystatin$_{dm}$ was approx. 1.5 LPS pg/μg protein, 0.03 endotoxin units (EU) per protein application.

6.1.6 Quantification of protein (BCA assay)

Protein concentrations were measured with the bicinchoninic acid test using BCA protein assay kit (Pierce, ThermoFisher Scientific, IL, USA) according to the manufacturer's instructions.

6.1.7 SDS-PAGE and Coomassie staining

Recombinant proteins were resolved on polyacrylamide gel by SDS-PAGE using the discontinuous buffer system (Laemmli, 1970) with and without reducing agent (β-mercaptoethanol, BME). For the production of polyacrylamide gels Bio-Rad apparatus was used (Bio-Rad Laboratories, Munich, Germany). The concentration of the acrylamide for the running gel was according to the molecular weight of the fractioned proteins (12-14%). For the stacking gel 6% acrylamide solution was used. The protein fractions (100 ng/well) were mixed with 2x sample buffer, heated for 3 min at 95°C. After loading the polyacrylamide gel, the samples were separated at 120-160V at RT for 1 h. To characterize the molecular weight of the fractionated proteins a standard with prestained proteins was used (New England Biolabs, Frankfurt, Germany). After the electrophoresis, separated protein fractions on the polyacrylamide gel were either stained for 30 min at 50-60°C with the Coomassie solution, destained and dried. Further immunological characterization, separated fractions were electrophoretically transferred into a nitrocellulose membrane (NC) (Towbin et al., 1979).

6.1.8 Inhibitor protease activity test

Cystatin was tested for cysteine proteinase inhibitor function in activity test to papain cleavage as described earlier (Hartmann et al., 1997). Briefly, rAvCystatin was incubated with papain and the substrate BAPNA for 30 min in 37°C. Test was read at 450 nm on ELISA plate reader (BioTek, Bad Friedrichshall, Germany) and inhibition of substrate cleavage by papain was calculated.

6.1.9 Analytical gel filtration and right angle static light scattering: oligomerization assay

Right angle static light scattering (RALS) system and refractive index detector (Malvern) were connected in line to an analytical gel filtration column Superdex 75 10/300 (GE Healthcare) to determine absolute molecular masses of the applied proteins. Molecular weight distributions were determined, thus the aggregations in each protein solution was assessed. To this end, 100 μl of 3-5 mg/ml unmodified rAvCystatin and rAvCystatin$_{tr}$ was applied and eluted through Superdex 75 10/300 column. The running buffer was PBS, pH 7.3 with a flow rate of 1 ml/min. Data were analyzed with the provided OmniSec software.

6.1.10 RNA and cDNA preparation

To prepare RNA from the intestinal tissue frozen segments were blended using a tissue homogenizer (MP Biomedicals, Eschwege, Germany) in RNA lysis buffer (Analytik Jena, Jena, Germany). Tissue supernatants were subsequently processed using the innuPREP RNA kit following the manufacturer's instructions (Analytik Jena, Jena, Germany).

6.1.11 Real time PCR

Real time PCR was used to analyse the expression levels of several cytokines at the residing site of helminths. 2 μg of RNA was reverse transcribed to cDNA using a High Capacity RNA to cDNA kit (Applied Biosystems, NJ, USA). The relative expression of β-actin, IL-25, IL-33 and TSLP was then determined via real time PCR using 5 ng of cDNA and FastStart Universal SYBR Green Master Mix (Roche, Zürich, Switzerland) and 300 nM primer pairs: **β-actin F1** 5' TCTTGGGTATGGAATCCTGTGGCA 3' and **β-actin R1** 5' TCTCCTTCTGCATCCTGTCAGCAA 3'; **IL-25 (IL-17E) F1** 5' ACAGGGACTTGAATCGGGTC 3' and **IL-25 (IL-17E) R1** 5' TGGTAAAGTGGGACGGAGTTG 3'; **IL-33 F1** 5' AGGAAGAGATCCTTGCTTGGCAGT 3' and **IL-33 R1** 5' ACCATCAGCTTCTTCCCATCCACA 3';

TSLP F1 5' AGCAAGCCAGCTTGTCTCCTG 3' and **TSLP R1** 5' TGTGCCATTTCCTGAGTACCGTCA 3' (TIB, Berlin, Germany) on AB7000 (Applied Biosystems, Darmstadt, Germany). Buffer conditions were used according to the manufacturer's instructions. PCR conditions: denaturation 10 min at 95°C; 40 cycles: 15 s at 95°C and 60 s at 60°C; 15 s at 95°C. Relative gene expression was determined via normalisation to the endogenous reference - the housekeeping gene (β-actin) and to strain specific naive controls using the 2-$\Delta\Delta$Ct method (Livak and Schmittgen, 2001). The mean values of sample triplets were then compared to corresponding controls.

6.2 Immunological methods

6.2.1 Western blot

Western blot analysis was used for the detection of proteins by polyclonal antibodies from rabbit serum raised against unmodified rAvCystatin. SDS-PAGE separated proteins were transfered into a NC at overnight at 80 mA. The NC membrane was then incubated in 5% non-fat dry milk/TBS for 1 h at RT to block the binding sites of the NC membrane. Afterwards the NC membrane was washed three times in 0.2% Tween20/TBS (TBST) while shaking. Primary antibody was applied for 2.5 h at RT. After the incubation, unbound antibodies were removed by washing the NC membrane 3 times with TBST. The secondary antibody (peroxidase-conjugated) was incubated with the NC membrane for 1.5 h at RT. Then, after washing 3 times with TBS, the color reaction was induced by addition of the corresponding substrate. To stop the reaction the NC membrane was washed with TBS. The NC membrane was then incubated with Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Freiburg, Germany) and the protein bands were detected by enhanced chemiluminescence (1 min in the dark) (GE Healthcare).

Antibodies (Ab):

- 1^{st} Ab: anti-rAvCystatin-rabbit serum, dilution 1:80 000 in 5% non-fat dry milk/TBS
- 2^{nd} Ab: Peroxidase-conjugated goat anti-rabbit IgG, dilution 1:2000 in 5% non-fat dry milk/TBS

6.2.2 Antibody detection

Total IgE and allergen-specific (OVA- and rPhl p 5b-) IgE, IgG1 and IgG2a levels in serum were determined by sandwich ELISA, as described before (Beier et al., 2004). For establishment of the grass pollen-induced model and comparison with an OVA-induced morel, kinetics of OVA- and Phl p 5b-specific antibodies was assessed

before sensitization on day 0, during the sensitization on day 12, before challenge on day 26 and after challenge on the day of the dissection (day 32). Standard sera using OVA were previously prepared as described earlier (Beier et al., 2004). For standard sera using grass pollen allergen BALB/c mice were injected i.p. with 5 μg rPhl p 5b and 2 mg Alum on days 1, 14, 21 and 39, followed by i.n. GPE (containing 9 μg/mg of rPhl p 5b, 6.09 ng/PNU as 38.5 μg/mg) challenge on days 53, 54, 55 and 62. Sera were obtained three days later. IgE standard (BD Pharmingen, Heidelberg, Germany) was used in accordance to samples to calculate total IgE amounts as μg/ml (dilution 1:2). Standard sera (for OVA-model #67, rPhl p 5b-model #1) were used to calculate amounts of allergen-specific IgE, IgG1 and IgG2a. For other experiments than kinetics evaluation, commercial IgG1 standard (BD Pharmingen, Heidelberg, Germany) was employed (dilution 1:1.5) to calculate specific IgG1 levels. Thus, in these experiments allergen-specific IgG1 were calculated as μg/ml and allergen-specific IgE and IgG2a were presented as lab units (LU). Table 6.1 shows dilutions of according Abs and sera used for immunoglobulin ELISAs performed in this study.

For biotynylation of allergens a 1 mg/ml solution of OVA grade VI (Sigma-Aldrich, Munich, Germany) or rPhl p 5b Batch#PP5br04001 (Allergopharma, Reinbek, Germany) (pH 8.3 with 100 mM $NaHCO_3$) were used. Allergens were incubated 1 h with 13.4 M sulfo-NHS-LC-Biotin at RT, in the dark shaking. Afterwards the solution was purified of unbound biotin by size exclusion chromatography (PD10-Sephadex column, GE Healthcare).

Table 6.1: Immunoglobulin detection: antibody and sera dilutions

Allergen	Immunoglobulin	1^{st} Ab, capture (concentration)	Standard serum	Sample naive	Sample immunized	2^{nd} Ab, detection (concentration)
rPhl p 5b	spec. IgE	a-IgE (3 μg/ml)	1:16	1:2	1:100	rPhl p 5b-Biot (0.5 μg/ml)
	spec. IgG1	rPhl p 5b (1 μg/ml)	1:131 072	1:5	1:400 000	a-IgG1-Biot 0.5 μg/ml
	spec. IgG2a	rPhl p 5b (2 μg/ml)	1:512	1:5	1:100	a-IgG2a-Biot (1 μg/ml)
OVA	spec. IgE	a-IgE (4 μg/ml)	1:32	1:2	1:100	OVA-Biot (3 μg/ml)
	spec. IgG1	OVA (10 μg/ml)	1:16 384	1:5	1:200 000	a-IgG1-Biot (0.5 μg/ml)
	spec. IgG2a	OVA (10 μg/ml)	1:256	1:5	1:100	a-IgG2a-Biot (2.5 μg/ml)

6.2.3 Cytokine detection

Animal models

Cell culture supernatants were harvested and tested for IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-13 and IFN-γ by cytokine ELISA kits (BD Biosciences, Heidelberg, Germany or eBioscience, CA, USA).

To assess the production of IL-25, IL-33 and TSLP in the intestinal tissue, frozen sections of small intestine were homogenized with tissue homogenizer (MP Biomedicals, Eschwege, Germany) in a buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany). Samples were centrifuged at 20 000g for 10 minutes and the total protein concentration in supernatants was determined via BCA test (Pierce, ThermoFisher Scientific, IL, USA). Samples were then assessed in duplicates via sandwich ELISA by DuoSet ELISA kit (R&D Systems, MN, USA) and the concentration (pg/ml) was normalized to total protein content (pg/mg total protein).

Human PBMCs

Cell-free PBMCs culture supernatants were collected and assayed for IL-4, IL-5, IL-10, IL-13 and IFN-γ by specific ELISA. Capture and detection Abs of IL-5 and IL-13 were purchased from BD Pharmingen (Heidelberg, Germany) and standards from Immunotools (Friesoythe, Germany). Detection Abs: 1 μg/ml for IL-5 and 3 μg/ml for IL-13. Capture Abs: 1 μg/ml for IL-5 and 0.5 μg/ml for IL-13. ELISA kits for IL-4, IL-10 and IFN-γ were ordered from eBioscience (CA, USA) and performed according to the manufacturers' instructions. The detection limit of the assay was 1.6 pg/ml for IL-4, 46.8 pg/ml for IL-5, 2.3 pg/ml for IL-10, 39 pg/ml for IL-13 and 3.9 pg/ml for IFN-γ. Optical density values of the samples were read at 450/570 nm on an ELISA plate reader (BioTek, Bad Friedrichshall, Germany).

6.2.4 Mast cell protease detection

At the day of dissection blood samples were collected and processed for sera. Sera from: naive, infected, allergic and allergic-AvCystatin-treated animals were assayed for mouse mast cell protease-1 (mMCP-1) by specific ELISA Ready-Set-Go kit (eBioscience, CA, USA) according to the manufacturer's instructions.

6.2.5 Proliferation assay

Animal models

Following MLN culture in 96-well plates with or without antigen stimuli, cellular proliferation was analysed via a further 20-hour culture in fresh media containing 1μCi methyl-[^{3}H]-thymidine (Amersham Pharmacia Biotech, Gent, Belgium) and subsequent detection using a β-counter (Perkin-Elmer, NY, USA). The uptake of labeled thymidine was measured by scintigraphy and showed proliferation rate in cpm (counts per minute).

Human PBMCs

In order to detect antigen-specific proliferation of PBMCs from allergic patients, cells were stained with CFSE (Carboxyfluorescein succinimidyl ester). CFSE passively diffuses into cells. It is colorless and non-fluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well retained and can be fixed with aldehyde fixatives e.g., formaldehyde. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away.

Freshly isolated human PBMCs (Subsection 6.6.2) were resuspended in PBS at 10x10^6 cells/ml. CFSE was diluted 1:1250 from a stock solution of 5 mM (stored at -20°C) just before use. Cells were mixed and incubated at 37°C for 5 minutes protected from the light. Reaction was stopped by washing the cells twice at 390g, 4°C, 15 minutes with RPMI-1640 medium containing 10% of autologous patient's serum. CFSE-labeled cells were washed, counted and resuspended in complete medium and cultured as described in Subsection 6.6.2.

6.2.6 Flow cytometric analysis

Animal models

After isolation of organs, single cell suspensions (minimum 1x10^6 cells) were stained with fluorescent conjugated antibodies against surface CD4, CD25 and intracellular stains for Foxp3, GATA-3, IL-13 or respective isotype controls. For intracellular cytokine staining 5x10^6 cells/well were stimulated with 50 ng/ml PMA and 1 μg/ml Ionomycin for 30 minutes, followed by further 3h incubation with Brefeldin A (10 μg/ml) (Sigma-Aldrich, Germany), which inhibits transport of proteins from endoplasmic reticulum (ER) to Golgi apparatus and induces retrograde protein transport to the ER. This leads to proteins accumulating inside the ER. After 3.5h-incubation,

cells were harvested, washed followed by a Fixation/Permeabilization Kit protocol (eBiosciences, CA, USA). Stained samples were acquired using FACSCanto, LSR II or Fortessa flow cytometers (BD Biosciences) and analyzed using FlowJo software (Tree Star, OR, USA).

Human PBMCs

Flow cytometric analysis was performed at day 7 of cultured CFSE-stained PBMCs. At day 7, 1x10^6 cells/well were incubated with 50 ng/ml PMA and 1 μg/ml Ionomycin (Sigma-Aldrich, Germany) for 1 hour, 37°C, 5% CO_2 and for last 4h in the presence of Brefeldin A (5 μg/ml) (Sigma-Aldrich, Germany). After 5h-incubation, cells were washed and resuspended in PBS. Cells were prepared for intracellular cytokine analysis according to the BD Cytofix/Cytoperm Kit protocol (BD Biosciences, Heidelberg, Germany) with modifications. For extracellular analysis on live cells, Fixable Viability Dye eFluor660 (eBioscience, CA, USA) was added for exclusion of dead cells. Briefly, cells were fixed in Fix/Perm solution (BD Biosciences) for 30 min, 4°C, washed and centrifuged in Perm Buffer (BD Biosciences), 390g, 10 min, 4°C. To avoid Fc receptor binding of fluorochrome-conjugated antibodies FcγR-blocking antibody (BD Biosciences) was used. Cells were washed again and subjected to FACS analysis. A total of 1x10^6 cells/treatment were intracellular stained with the following antibodies: Alexa Fluor 405-conjugated anti-human CD4 (to circumvent PMA-induced loss in surface CD4 on human T cells (Acres et al., 1986; Bigby et al., 1990), PeCy7- conjugated anti-human IL-4, Alexa Fluor 430-conjugated anti-human IFN-γ (all as a gift from DRFZ, Berlin, Germany), PE-conjugated anti-human IL-10 (BD Biosciences). Stained cells were acquired on a FACSCanto flow cytometer (BD Biosciences) with FACS-DIVA software and further analyzed using FlowJo software (Tree Star, OR, USA).

6.3 Parasitological methods

6.3.1 *Heligmosomoides polygyrus* and *Trichuris muris* life cycle and infection

In frame of this work two helminth infections with *H. polygyrus* and *T. muris* in mouse were used. To further understand the aspects of immune response, life cycles of both parasites are briefly introduced here.

H. polygyrus (*Hp*) infection is an example of efficient parasitism. Once the infection is established, becomes chronic and lasts weeks to months in genetically competent strains of laboratory mice. The free-lining larvae (L1) hatches from the egg in the

environment and undergoes two molts. L3 stage is infectious by oral ingestion. It moves into the gastric mucosa of the stomach as early as 4 hours after inoculation, emerges 36 hours later, enters the duodenum and lies next to the longitudinal muscle layer of the gut. The third molt is 2 days post infection, the fourth molt 4 to 6 days later, and adults appear at day 8 p.i. into the lumen, where they mate and produce eggs. From day 9 p.i. eggs are passed in the feaces and continue to appear for several days (reviewed in Donskow-Schmelter et al., 2009).

T. muris (*Tm*) is transmitted via the ingestion of embryonated eggs that pass through the digestive tract. After reaching ceacum, eggs are hatching and the first stage larvae (L1) appears. L1 penetrates the mucosal layer and epithelium of ceacum, where it grows and undergoes molting at day 9, 17 and 22 p.i. At day 32 p.i. it reaches maturity, migrates into the lumen where the adult worms mate and release eggs into the host feaces (reviewed in Cliffe and Grencis, 2004).

In frame of this work mice were in infected with *H. polygyrus* via oral gavage with 200 infective L3 in 200 μl distilled H_2O. Infection with *T. muris* was performed with 200 embryonated eggs via oral gavage. Adult parasite burdens were assessed via longitudinal dissection of either small intestine (*Hp*) or the caecum and proximal colon (*Tm*) and counted using a binocular microscope.

6.3.2 Fecal egg count

The shedding of *H.polygyrus* eggs was assessed via collection and weighing of murine faecal pellets at days 14, 16 and 18 p.i. from each mouse. Faeces were suspended in 1 ml distilled H_2O. Saturated NaCl solution (5 ml) was added, mixed and 650 μl of the solution was placed into MacMaster chambers. Eggs were counted in duplicated via light microscopy.

Total eggs per gram (g) faeces were calculated by the following formula:

$$\frac{\text{Number of eggs in sample} \times \text{sample volume} \times 6.67}{\text{faeces weight in g}}$$

Counts were expressed as the median counts of eggs enumerated on three independent days per mouse.

6.3.3 *H. polygyrus* adult worm antigen

Worm antigen was prepared from *H. polygyrus* adult worms incubated for 24h in RPMI-1640 medium containing 100 U/ml penicillin and 100 μg/ml streptomycin. Worms were homogenized and sonicated (1 min, 60W) on ice in PBS, followed by centrifugation for 20 min, 20 000g at 4°C). For sterilization, the antigen extract was filtered through a 0.4 μm filter and stored at -80°C until usage.

6.4 Protocols for animal models

Female BALB/c and C57BL/6 were purchased from Charles River (Sulzfeld, Germany) and either housed at the animal facilities of the Charité-University Medicine Berlin Campus Virchow-Klinikum or Humboldt University Berlin. MC-deficient mice Kit^{W}/Kit^{W-v}; Kit^{W-sh}/Kit^{W-sh} and WBB6 background control (wild type, WT) strain mice were bred and housed at the animal facilities of the Charité-University Medicine Berlin Campus Mitte. All mice were handled following national guidelines and experiments were approved by the animal ethics committee.

6.4.1 MC-deficiency and worm infection

Infection of MC-deficient (Kit^{W}/Kit^{W-v}; Kit^{W-sh}/Kit^{W-sh}) and WBB6 background control (WT) strain mice with *H. polygyrus* and *T. muris* was performed as described in Subsection 6.3.1.

At the day of the dissection following analyses were performed:

1. removal of spleen, MLNs to perform cell culture, proliferation and FACS analyses
2. removal of parts of intestine for adult worm counts
3. cryoconservation of parts of intestine to perform tissue homogenates for real time PCR analyses
4. preparation of intestinal sections for histological analyses
5. collection of blood to monitor antibody production and levels mMCP-1 in serum

6.4.2 rPhl p 5b- vs. OVA-induced model of allergy and treatment with AvCystatin

Female BALB/c mice, 8-week-old mice were sensitized three times (day 1, 14 and 21) i.p. with 5 μg rPhl p 5b (Genbank acc. no. Q40963, gift from Allergopharma, Reinbek, Germany) emulsified in 2 mg of aluminum hydroxide, Alum (Imject Alum, Pierce, ThermoFisher Scientific, IL, USA) for the rPhl p 5b-induced model. For the OVA-induced model, animals were injected two times (day 1 and 14) i.p. with 20 μg OVA (Grade VI, Sigma-Aldrich, Germany) emulsified in 2 mg Alum. Subsequently, on days 28 and 29, mice were anesthetized by an i.p. injection of ketamine/rompun (Bayer and Bela-Pharm, Berlin, Germany) and challenged twice i.n. with grass pollen extract (GPE) (Allergopharma, Reinbek, Germany) containing 9 μg Phl p 5b (6.09 ng/PNU as 38.5 μg/mg) (rPhl p 5b-induced model) or with 50 μg OVA (OVA-induced model). Scheme of both models is presented in Fig. 3.9.

Sensitized with rPhl p 5b mice were treated i.p with 20 μg unmodified rAvCystatin, rAvCystatin$_{mut}$, rAvCystatin$_{tr}$ or the control protein rDHFR on four occasions, on days 1, 7, 14 and 21 (during the sensitization phase) and challenged i.n. with GPE on days 28 and 29 (Fig. 3.11).

Naive controls were injected with Alum in PBS and challenged under anaesthesia with PBS.

Airway reactivity (AR) was measured *in vivo* on day 31 (described in Subsection 6.4.4).

Analyses were performed one day after AR measurement and included:

1. lavage of lungs to collect BAL fluids to assess levels of cytokines, cell numbers and types
2. removal of PBLNs and spleen to cell cultures and/or FACS analyses
3. preparation of lungs for histological analysis (formalin)
4. collection of blood to obtain sera for assessment of antibody production and levels of mMCP-1

6.4.3 Model of allergen-specific immunotherapy (SIT) and AvCystatin

Female BALB/c mice, 8-12-week-old (Charles River, Sulzfeld, Germany) on day 1 and 14 received i.p. sensitizations with 20 μg OVA emulsified in 2 mg of Alum. Aerosol challenges with 1% OVA in PBS (1 g in 100 μl) were applied on days 42, 44 and 46. The allergen-specific immunotherapy (SIT) treatment was performed s.c. with 1 mg (SIT high) or 100 μg (SIT low) OVA between sensitization and challenge phase days 28, 30 and 32 (Fig. 3.23).

Cystatin was applied after sensitization and before challenge: on days 28, 30 and 32 rAvCystatin was mixed beforehand with OVA and applied together s.c. (rAvCystatin + OVA s.c.) as a therapy in the first approach. In the second and third setup, it was administered on days 28, 30 and 32 in two separate injections either s.c. or i.p. at the same time with OVA s.c. (SIT) (rAvCystatin s.c. & OVA s.c. or rAvCystatin i.p. & OVA s.c.) (Fig. 3.25). In the last approach rAvCystatin was applied i.p. alone after allergen sensitization and before challenge on days 28, 30 and 32 (Fig. 3.25). One day before analysis, AR was measured *in vivo* (day 49, Subection 6.4.4).

Analyses on day 50 included:

1. lavage of lungs to collect BAL fluid to assess levels of cytokines, cell numbers and types
2. removal of PBLNs, spleen to cell cultures and/or FACS analyses or proliferation
3. preparation of lungs for histological analysis (formalin)
4. collection of blood to obtain sera for assessment of antibody production

6.4.4 Measurement of airway reactivity (AR)

Airway responsiveness was determined one day before dissections by whole body plethysmography in unrestrained animals to inhaled increasing doses of metacholine (MCh) (Sigma-Aldrich, Germany) as described elsewhere (Hamelmann et al., 1997b). Baseline was established in response to PBS inhalation. The mean values of the Penh (enhanced pause) for every MCh concentration were calculated. Index Penh values of individual animals (Penh value of MCh minus baseline) were analysed as mean values of each group.

6.4.5 Bronchoalveolar lavage (BAL) fluid

One day after AR measurements, mice were sacrificed, blood samples were collected and BAL fluids harvested by flushing the lung airways via the trachea two times with ice cold 0.8 ml suspension of protease inhibitor cocktail (Roche, Mannheim, Germany) in PBS. The tubes were weighted and BAL fluid from the first lavage was centrifuged (10 min at 2 000 rpm, 4°C), supernatants were collected and stored at -80°C for subsequent analysis of cytokines.

BAL cells were recovered and combined from both lavages, resuspended in PBS and counted (total cell number). 20 μl aliquot was stained with Kimura stain and MCs were counted via light microscopy. 100 μl of remaining cells was spun down using a cytospin centrifuge for staining and differentiation (ThermoShandon Cytospin 4, Frankfurt, Germany). Dried grass slides with cytospined cells were stained with DiffQuick (Fisher Scientific, Germany) according to the manufacturer's instructions.

The numbers of eosinophils, lymphocytes and macrophages were differentiated by morphological criteria (count of 200 cells/slide under light microscopy in a blinded fashion), as previously described (Blumchen et al., 2006).

6.4.6 Histological analyses

Lungs were fixed in 3.7% phospate-buffered formalin, embedded in paraffin wax, 2 μm sections were cut and stained using standard histological protocols with Hematoxylin and Eosin (H&E) for histomorphological evaluation, with periodic acid-Schiff reagent (PAS) for carbohydrates detecting goblet cells and Toluidine blue for MCs. Sections were evaluated via light microscopy.

6.4.7 Reconstitution with rIL-25

In some experiments with MC-deficient mice, Kit^{W}/Kit^{W-v} and WBB6 mice strains, on days 1-4 p.i. with *Hp*, animals received i.p. 0.4 μg rIL-25 in PBS (R&D Systems, MN, USA).

6.4.8 Reconstitution with bone marrow

In some experiments with MC-deficient mice, Kit^{W}/Kit^{W-v} mice were reconstituted with $Kit^{+/+}$ (WT) bone marrow via intravenous transfer of $1x10^{7}$ purified mononuclear bone marrow cells. Reconstituted animals rested for 6 weeks prior to parasite infection.

6.5 Recruitment criteria for allergic subjects

Allergic patients were recruited via an announcement at the Charité Campus Mitte, Berlin, e-mail or telephone contact directly by the study nurse. The blood samples (up to 40 ml) from 3 allergic subjects and 2 healthy controls were taken by the study nurse once out of the pollen season and from 21 allergic patients once in the season. Patients signed in the written consent. The whole study was monitored by the study doctor and approved by the local Ethics Committee (Charité-University Medicine Berlin).

Inclusion/exclusion criteria were set up for the recruitment of allergic patients recruited in the pollen season. Some patients did not strictly meet the criteria concerning fixed class of allergen-specific IgE. However, all of them were allergic to timothy grass pollen and because of the low sample size, all of them were considered in the study. The subgroup analysis of the results was further performed.

Inclusion criteria were following:

1. Positive test: ImmunoCAP™ class 3 or higher to sweet vernal grass (*Anthoxanthum odoratum*), timothy grass (*P. pratense*) and rye (*Secale cereale*), high titer of allergen-specific IgEs

2. Clinical symptoms and documented history of grass pollen allergy
3. No oral steroid therapy or immunotherapy received at least 3 months before the study

Exclusion criteria:

1. Negative test: ImmunoCAP™ class 0 to sweet vernal grass (*A. odoratum*), timothy grass (*P. pratense*) and rye (*S. cereale*), no allergen-spec. IgE detected
2. No clinical symptoms or history of grass pollen allergy
3. Oral steroid therapy or immunotherapy received not later than 3 months before the study
4. Illnesses within the past two weeks or any chronic or acute medical problems apart from allergy that would influence immune parameters
5. Receiving any kind of suppressive medications

Twenty-one allergic patients (age 21-49; eight males and thirteen females) with clinical symptoms and history of grass pollen allergy were examined during the pollen season. Patients were verified for allergen-specific IgE to three common grass pollen allergens: sweet vernal grass (*A. odoratum*), timothy grass (*P. pratense*) and rye (*S. cereale*). All asthmatic patients had allergen-specific IgE to grasses detected in the serum (>0.35 kU/l, class >1) (ImmunoCAP™ System, Phadia AB, Uppsala, Sweden). For the establishment experiment three allergic subjects with serum IgE class three and two healthy controls with no history of allergic symptoms and negative (<0.35 kU/l, class 0) to common grass allergens, were examined out of the pollen season. On entering the study, subjects were free of oral steroids or immunotherapy for at least 3 months. All patients were in a good health conditions. However, in the written questionnaire five of them reported atopic dermatitis, one communicated urticaria and one high blood pressure. Clinical characteristics of allergic subjects are presented in Table 3.5, Section 3.5

6.6 Cell culture techniques

6.6.1 Preparation of organs for cell culture

Mouse models of asthma, MC-deficiency and helminth infection

Spleens, PBLNs and MLNs of mice were passaged under sterile conditions via through a 70 μm nylon mesh. If necessary, erythrocytes were removed by resuspension of washed cells in erythrocyte lysis buffer for 5 min on ice. Cells were washed twice and resuspended in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM L-glutamin, and 5-10% fetal calf serum (FCS) (HyClone, Perbio Science, ThermoFisher, Germany) and cultured (MC-deficiency model:

1x10^6 cells/well and asthma model: 3.5x10^5 cells/well) for 48 or 96 h (MC-deficiency and asthma model, respectively) in 5% CO_2 at 37°C. Cells for FACS analysis after isolation were directly resuspended at least 1x10^6 cells/staining and analysis was processed. For intracellular cytokine staining were resuspended 5x10^6 cells/well and handled as described in Subsection 6.2.6.
Otherwise, cell culture supernatants were stimulated as indicated below, cultured for a given time and harvested. Supernatants were tested for cytokines as described in Subsection 6.2.3.

Stimulations of cells were following:

Asthma model

- RPMI-1640 alone
- 2.5 μg/ml ConA
- 20 μg/ml OVA (OVA-model)
- 5 μg/ml rPhl p 5b (rPhl p 5b-model)
- 10 μg/ml cystatins (unmodified rAvCystatin, rAvCystatin$_{mut}$, rAvCystatin$_{tr}$)

MC-deficiency and helminth infection model

- RPMI-1640 alone
- 20 μg/ml *H. polygyrus* antigen (*Hp*-Ag)
- 50 μg/ml *T. muris* E/S (*Tm* E/S-Ag)
- 1 μg/ml anti-CD3 and anti-CD28 mAb

SIT model

Spleen and PBLNs were isolated aseptically and organs were passed through through a 70μm nylon mesh under sterile conditions. If necessary, erythrocytes were removed by resuspension of washed cells in erythrocyte lysis buffer for 5 min on ice. Cells were washed twice. Spleen mononuclear cells were isolated by density gradient centrifugation (Lympholyte-M, Cedarlane Laboratories, Ontario, Canada). Spleen mononclear cells and PBLNs were resuspended in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM L-glutamin, and 10% fetal calf serum (FCS) (HyClone, ThermoFisher, Bonn, Germany) and cultured for 96 h (MC-deficiency and asthma model, respectively) in the presence of RPMI-1640 media alone, 2.5 μg/ml ConA, 50 μg/ml OVA or 10 μg/ml rAvCystatin in 5% CO_2 at 37°C. Cells for FACS analysis after isolation were immediately resuspended at least 1x10^6 cells/staining and analysis was performed as described in Subsection 6.2.6. Otherwise, cell culture supernatants were harvested and stored at -20°C until performance of cytokine ELISA as described in Subsection 6.2.3.

6.6.2 Isolation and culture of human PBMCs

Blood was collected by a certified study nurse into tubes containing anticoagulant (Lithium Heparin) (BD, Heidelberg, Germany) and centrifuged at 700g, RT for 5 minutes to isolate patients' plasma. Plasma was collected and further centrifuged at 1400g in order to remove remaining blood cells. The upper fraction without cells was collected and used as autologous patient's sera. Remaining red blood fraction was diluted with an equal volume of RPMI-1640 medium, carefully layered and centrifuged (800g, RT, 20 minutes) over Lymphoprep™ density gradient (Axis-Shield PoC AS, Oslo, Norway). After centrifugation the mononuclear cells that formed a distinct band at the sample/medium interface were removed from the interface using a Pasteur pipette without removing the upper layer. The harvested fraction was diluted with RPMI-1640 medium to reduce the density of the solution and the cells were washed at 600g, 4°C, 15 minutes. Supernatant was aspirated and in order to remove the platelets, the cells were pelleted one more time by centrifugation for 15 minutes at 250g, 4°C. Isolated and washed PBMCs were resuspended at $2x10^6$ cells/ml in RPMI-1640 culture medium, 5% patient autologus serum, 1% L-glutamine (2 mM), 1% penicillin (100 U/ml)/ streptomycin (100 mg/ml), 0.1% β-mercaptoethanol (BME) and incubated for 3 days (for supernatants) or for 7 days (for surface marker and intracellular cytokine staining) in 24-well plate at the density of $1x10^6$ cells/well. In all experiments viability of isolated PBMCs was over 95%.

Dose and time response experiments with GPE

The titration experiment with three timothy grass pollen allergic patients and two healthy controls was performed in order to define the dose and timing of GPE used for an *in vitro* restimulation of PBMCs. Cells were incubated with different concentrations range: 2, 5, 10 and 20 μg/ml of GPE (Allergopharma, Reinbek, Germany) for 7, 9 and 11 days in conditions described in Subsection 6.6.2. The GPE-concentration and time inducing the best proliferative response before an *in vitro* treatment with rAvCystatin was determined and used for evaluating the course of the T cell reactivity to GPE during the study period.

Dose and timing of incubation with AvCystatin

The titration experiment with three timothy grass pollen allergic patients and two healthy control was performed out of the pollen season in order to define the dose and timing of incubation with rAvCystatin in an *in vitro* restimulation of PBMCs. Cells were incubated with different concentrations (2.5 and 20 μg/ml) of rAvCystatin that was either added 2 hours prior to or at the same time of stimulation with 5

μg/ml GPE. Cells were incubated for 3, 5, 7 and 9 days in conditions described in Subsection 6.6.2. The concentration and incubation time with rAvCystain showing the strongest suppressive capacity was determined and used in experiments in the pollen season.

6.7 Statistical analysis

Each animal experiment was performed with 4-6 animals per group, and the experiments shown are representative of three independent experiments unless otherwise stated. Experiments with human PBMCs were performed once out and once in the grass pollen season. Patient number was indicated in each experiment. Statistical analysis was performed with the unpaired Student's t test, Kruskal Wallis analysis or 1-way ANOVA where applicable. Data are presented as means ±SEM. Values of *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$ were considered statistically significant.
The Spearman's correlation coefficient test was used to determine the correlation among different variables. The line fitting results was determined using linear regression analysis. Differences were defined as statistically significant for $p< 0.05$.

7. Materials

7.1 Biological resources

E. coli DH5α	Invitrogen, Darmstadt, Germany
E. coli BL21 (DE3)	Novagen, Merck KGaA, Darmstadt, Germany
pET28b(+)	Novagen, Merck KGaA, Darmstadt, Germany
BALBc and C57BL/6 mice	Charles River, Sulzfeld, Germany
Kit^{W}/Kit^{W-v}; Kit^{W-sh}/Kit^{W-sh} mice	Charité , Berlin, Germany
WBB6 control (WT) mice	Charité, Berlin, Germany

7.2 Laboratory equipment

Bio-Rad apparatus	Bio-Rad, Munich, Germany
Centrifuges	Eppendorf, Hamburg, Germany
Cytocentrifuge (cytospin)	ThermoShandon 4, Frankfurt, Germany
ELISA-reader	BioTek, Bad Friedrichshall, Germany
FastPrep-24 Homogenizer	MP Biomedicals, Eschwege, Germany
Flow cytometer: LSRII, FACSCanto	BD Biosystems, CA, USA
Real-time PCR system	7300 Applied Biosystems, CA, USA
Scintillation-spectroscope	Trilux 1450 Wallac, Turku, Finland
Square wave electroporator	BTX ECM830, BTX, Holliston, USA
Ultrasound-disintegrator	Heinemann, Schw. Gmünd, Germany
Whole-body plethysmograph (AHR)	emka technologies, Paris, France

7.3 Buffers and media

Coomassie	20% EtOH 10% acetic acid 2 tabl. PhastGel BlueR (Amersham) up to 1 l dest. H_2O
Destaining buffer	20% EtOH 10% acetic acid
Activity test buffers	0.2 M K_2HPO_4 0.2 M KH_2PO_4 0.004 M DTT 0.004 M EDTA 0.01% (v/v) Brij
Transfer buffer	50 mM Tris 380 mM Glycin 0.1% SDS 20% Methanol
10x TBS	1 M NaCl 50 mM Tris, pH 7.5
TBST	0.2% Tween20 1x TBS
LB-Medium	10 g NaCl 10 g Trypton 5 g Yeast extract add 1 l mit H_2O, pH 7.5 autoclaved
LB-Plates	LB-Medium 1.5 % Agar autoclaved
SOB-Medium	20 g Trypton 5g Yeast extract 0.5 g NaCl 10 ml KCl (250 mM) add 995 ml H_2O pH 7.0 5 ml sterile 2 mM $MgCl_2$ autoclaved
SOC-Medium	SOB-Medium 10 mM filtered glucose
Erythrocyte lysis buffer	0.01 M $KHCO_3$ 0.155 M NH_4Cl 0.1 mM EDTA pH 7.5
Cell culture medium (animal cells)	RPMI-1640 5-10% FCS 2 mM L-glutamine 100 U/ml penicillin 100 μg/ml streptomycin

Cell culture medium (human cells)	RPMI-1640 5% patient autologus serum 2 mM L-glutamine 100 U/ml penicillin 100 μg/ml streptomycin 0.1% β-mercaptoethanol all from Biochrom, Berlin, Germany
BAL-wash-solution	10 ml PBS (Dulbecco's w/o) 1 tabl. complete protease inhibitor (Roche)
Lympholyte-M	Cedarlane Laboratories, Ontario, Canada
Lymphoprep™	Axis-Shield PoC AS, Oslo, Norway
FACS staining buffer	0.2% BSA in PBS
FACS fixation buffer	0.2% BSA, 0.5% paraformaldehyde (PFA) in PBS

7.4 Protein purification buffers

PBS	137 mM NaCl 2.7 mM KCl 8 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4
rDHFR:	
Lysis buffer	300 mM NaCl 50 mM NaH_2PO_4 20 mM Imidazol pH 7.4
Wash buffer	300 mM NaCl 50 mM NaH_2PO_4 30 mM Imidazol pH 8
Elution buffer	300 mM NaCl 50 mM NaH_2PO_4 250 mM Imidazol pH 8
rAvCystatin:	
Lysis buffer	PBS/0.1% (v/v) Triton, pH 8
Wash buffer	PBS/10% (v/v) Glycerin, pH 5.8
Elution buffer	PBS/10% (v/v) Glycerin, pH 3

Neutralization buffer	PBS, pH 10
Dialysis 1	PBS, pH 7.4 or PBS/0.05% (v/v) Triton, pH 7.4
Dialysis 2	PBS, pH 7.4 or PBS/0.05% (v/v) Triton, pH 7.4

7.5 Chemicals and biologicals

1μCi methyl-[^{3}H]-thymidine	Amersham Pharmacia Biotech, Gent, Belgium
Alum (Imject Alum)	Pierce, ThermoFisher Scientific, IL, USA
BAPNA (92 μM)	Bachem, Weil a. Rhein, Germany
BSA, fraction V	AppliChem, Darmstadt, Germany
mCD3	eBioscience, CA, USA
mCD28	eBioscience, CA, USA
CFSE	eBioscience, CA, USA
ConA	Sigma-Aldrich, Germany
Cytokine standards	Immunotools, Friesoythe, Germany
Fetal Calf Serum (FCS)	HyClone, ThermoFisher, Bonn, Germany
GPE Batch#177CHB W8000480	Allergopharma, Reinbek, Germany
Ketamine/rompun	Bayer and Bela-Pharm, Berlin, Germany
rIL-25	R&D Systems, MN, USA
Metacholine (MCh)	Sigma-Aldrich, Germany
OVA grade VI	Sigma-Aldrich, Munich, Germany
Papain (100 μM)	Sigma, Munich, Germany
Paraformaldehyde	Sigma, Munich, Germany
PhastGel BlueR	AmershamPharmacia, Uppsala, Sweden
rPhl p 5b Batch#PP5br04001	Allergopharma, Reinbek, Germany
Phusion Hot Start DNA Polym.	New England Biolabs, Frankfurt, Germany
PMA, Ionomycin, Brefeldin A	Sigma, Munich, Germany
Proteinase inhibitor cocktail	Roche, Mannheim, Germany
Quick T4 DNA Ligase	New England Biolabs, Frankfurt, Germany

7.6 Commercial kits

Amersham ECL Plus WB Detection	GE Healthcare, Freiburg, Germany
BCA kit	Pierce, ThermoFisher Scientific, IL, USA
BD Cytofix/Cytoperm Kit	BD Biosciences, Heidelberg, Germany
Cytokine bead array flex sets	BD Biosciences, Heidelberg, Germany
DiffQuick	Fisher Scientific, Germany
DuoSet ELISA kit	R&D Systems, MN, USA
ELISA kit	eBioscience, CA, USA
ELISA Ready-Set-Go kit	eBioscience, CA, USA
EndoTrap system blue	Profos, Regensburg, Germany
Fixation/Permeabilization kit	eBiosciences, CA, USA
High Capacity RNA to cDNA kit	Applied Biosystems, NJ, USA
ImmunoCAP™	System Phadia AB, Uppsala, Sweden
innuPREP RNA kit	Analytik Jena, Jena, Germany
LAL QCL-1000	Cambrex, BioSciences, Walkersville, USA
OptEIA ELISA kit	BD Biosciences, Heidelberg, Germany
Phusion kit	Finnzymes, Espoo, Finnland
Plasmid-Mini-Kit	Qiagen, Hilden, Germany
Primers	TIB, Berlin, Germany
SYBR Green Master Mix	Roche, Zürich, Switzerland
QIAshredder spin columns	Qiagen, Hilden, Germany

7.7 Antibodies and secondary reagents

anti-mIgE	BD Pharmigen, Heidleberg, Germany
anti-mIgG1-biotinylated	BD Pharmigen, Heidleberg, Germany
anti-mIgG2a-biotinylated	BD Pharmigen, Heidleberg, Germany
FcγR-blocking Ab	BD Biosciences, Heidelberg, Germany
Fixable Viability Dye eFluor660	eBioscience, CA, USA
anti-mCD4 e450	eBioscience, CA, USA
anti-mCD25 Alexa700	eBioscience, CA, USA
anti-mCD103 Bio-SA-Cy7	eBioscience, CA, USA
anti-mF4/80 Cy5	eBioscience, CA, USA
anti-m/hFoxp3 PE	eBioscience, CA, USA
anti-mGATA-3 APC	eBioscience, CA, USA
anti-mIL-13 PE	eBioscience, CA, USA
anti-hCD4 Alexa Fluor 405	a gift from DRFZ, Berlin, Germany
anti-hIL-4 PeCy7	a gift from DRFZ, Berlin, Germany
anti-hIL-5, anti-hIL-13 ELISA Abs	BD Pharmingen, Heidelberg, Germany
anti-hIL-10 PE	BD Biosciences, Heidleberg, Germany
anti-hIFN-γ Alexa Fluor 430	a gift from DRFZ, Berlin, Germany

7.8 Software

BD FACSDiva	BD Biosciences, Heidelberg, Germany
FCAP Array Software	Applied Biosystems, Darmstadt, Germany
FlowJo	Tree Star, OR, USA
PrimerExpress	Applied Biosystems, Darmstadt, Germany
Prism	GraphPad Software, CA, USA
SPSS Statistics	IBM Corporation, NY, USA

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Publications List

Written in the course of this work:

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Congress Contributions

Presentations (since 2010)

1. **Daniłowicz-Luebert E.**, Lucius R., Hamelmann E. and Hartmann S. Filarial immunomodulator alters grass allergic responses. *European Academy of Allergy and Clinical Immunology (EAACI) Congress 2012*, 16-20 June 2012, Geneva (Switzerland).
2. **Daniłowicz-Luebert E.**, Lucius R., Hamelmann E. and Hartmann S. A nematode cystatin modulates murine and human allergic responses to grass. *Joint Meeting of German Society for Tropical Medicine and International Health (DTG) and German Society for Parasitology (DGP)*, 14-17 March 2012, Heidelberg (Germany).
3. **Daniłowicz-Luebert E.**, Lucius R., Hamelmann E. and Hartmann S. Influence of a filarial cystatin on a clinically relevant model of allergic asthma. *EAACI/GA²LEN Allergy School*, 15-18 September 2011, Edinburgh (UK).
4. **Daniłowicz-Luebert E.**, Hepworth M.R., Rausch S., Metz M., Maurer M. and Hartmann S. Role of mast cells in early Th2 induction during nematode infection. *Berlin Immunology Day, Research Center ImmunoSciences*, 9 December 2010, Berlin (Germany).
5. **Daniłowicz E.**, Hamelmann E., Lucius R. and Hartmann S. A parasitic cystatin interferes with a clinically relevant model of grass pollen induced allergic airway hyperreactivity. *12th MDC/FMP/HSR PhD Retreat*, 9-11 September 2010, Rheinsberg (Germany).
6. **Daniłowicz E.**, Hamelmann E., Klotz C., Lucius R. and Hartmann S. Interference of a parasitic immunomodulator with a clinically relevant model of airway hyperreactivity. *Joint Meeting of the German Societes of Parasitology and Protozoology at Düsseldorf University*, 17-20 March 2010, Düsseldorf (Germany).
7. **Daniłowicz E.**, Hamelmann E., Klotz C., Lucius R. and Hartmann S. Treatment of allergic airway hyperreactivity with filarial cystatin. *4th short Course for Young Parasitologists*, 13-16 March 2010, Düsseldorf (Germany).

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1. **Daniłowicz-Luebert E.**, Lucius R., Hamelmann E. and HartmannS. Parasitic immunomodulator and its mutated form ameliorate allergic airway hyperreactivity. *4th International Symposium of Molecular Allergology (ISMA 2010)*, 29-31 October 2010, Munich (Germany).
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Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Berlin, den 06.09.2012 Emilia Daniłowicz-Luebert